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FREEZING AND DRYING OF BIOLOGICAL MATERIALS

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Consulting Editor and Conference Chairman
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Part I. Physical Aspects of Freezing

GENERAL PRINCIPLES OF FREEZING AND FREEZING INJURY IN CELLULAR MATERIALS

Harold T. Meryman

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The acquisition of knowledge appears to pass through four stages. The first is the stage of total ignorance in which observations have no significance beyond themselves and cannot be integrated into a general theory. The second stage is one of simplification in which a general theory is developed that is both simple and reasonable and appears to fit experimental observations except for a few that we presume to be in error. During the third stage conflicting observations are reconfirmed and new experiments carried out that do not fit the simplified theory. Exceptions to the theory grow in number, controversies develop, and confusion is re-established. The fourth stage, based on more exacting research and extensive experience, evolves order from chaos and understanding from confusion. This final stage is often unattainable.

Cryobiology is now in the second of these stages. A simple descriptive theory has been developed that fits most of the experimental facts and ignores only a few.[1] We are pleased with ourselves for having constructed a model so simple that we feel confident of its ability to explain any observation, even though it may have been unpredictable. What I am about to present, then, is the simple, authoritative, pre-emptory, infallible theory of the second phase of our understanding of the mechanism of freezing and of freezing injury in biology. Where there are shortcomings I shall ignore them for the moment since, despite its ultimate fallibility, the model I shall depict works well for most purposes. Inasmuch as most investigators are not intimately familiar with biological freezing, they will be better served initially by simplification. The papers to follow examine various aspects of the system in greater detail, and it will rapidly become evident in what areas we must modify our ideas. This, in effect, is the underlying purpose of this monograph: to state, to test, and to modify our understanding of the basic mechanisms involved in freezing and in drying, avoiding as much as possible the introduction of specific applications except as they contribute to an understanding of the general mechanism.

This monograph is published at a rather significant period in the history of research on low-temperature biology. Despite man's long association with cold, research on the subject has consisted primarily in scattered studies of the resistance or susceptibility of various organisms to injury from freezing. Only in the last twenty or thirty years has real progress been made toward understanding what is involved in freezing injury and toward means of circumventing this injury to permit the survival of living cells. The researches of Luyet and Gehenio[2] in the area of ultrarapid freezing and the discovery of the protective qualities of glycerin by Polge *et al.*[3] have provided a great impetus to further research and to applications of freezing. With the development of means for preventing the otherwise inevitable destruction of living cells by freezing, our understanding of the mechanism of injury has been expanded to the extent that a reasonably satisfactory hypothesis can now be presented.

Probably the most fundamental and unimpeachable statement that one can make regarding freezing of biological materials is that it is basically a physical phenomenon. For all practical purposes there are two dominant effects produced by lowering the temperature of a tissue or a cell suspension. The first of these is a reduction in the rate at which physical and chemical phenomena take place. The extent to which various reactions slow down at low temperature varies depending upon the reaction; some complex phenomena, such as nerve conduction, cease altogether at room temperatures not far below their normal operating range. Other simpler chemical reactions continue to take place at a reduced rate even at surprisingly low temperatures. Serious metabolic dislocation can occur due to an imbalance between the rate at which interdependent processes may occur. For example, in the intact mammal cooled to a few degrees above freezing, respiration and circulation will cease altogether, although tissue metabolism is still continuing at a reduced rate, and these tissues may be injured irreversibly by anoxia as a result. Consequently, it appears that injury from temperature reduction alone is due to dislocations of the metabolic production line and cannot in the strictest sense be ascribed to any direct effect of reduced temperature.

The second dominant phenomenon associated with a lowering of temperature is phase change. The most familiar and biologically most significant example is the freezing of water, which consists in a change of phase from liquid to crystalline solid. Many organic materials, such as fats, do not undergo so specific a transition, but merely increase in viscosity, approaching and reaching rigidity at lowered temperature. The formation of ice from water is pre-eminently the most important physical change occurring when tissue is carried to low temperature.

The first step in the development of an ice crystal is the formation of a crystal nucleus of critical size. A crystal nucleus is an aggregation of molecules that may grow to form a larger crystal. It is said to be of critical size when there is an equal probability that it may grow or vanish. Due to energy considerations that need not be considered here, critical size is temperature-dependent. At higher temperatures critical size is very large and is smaller as temperature is reduced. At temperatures near the freezing point, critical size is so large that it is highly improbable that a nucleus could result from random aggregations of water molecules. In this range, nucleation is presumed to occur on inclusions that provide a substitute nucleus about which further growth may take place. This process is referred to as heterogeneous nucleation. Even this type of nucleation is relatively improbable at the freezing point so that supercooling, the lowering of temperature below the freezing point without crystallization, is commonly seen when relatively small volumes are cooled. The probability for the existence of a heterogeneous nucleus of critical size improves with further lowering in temperature. Homogeneous nucleation probably does not occur until quite low temperatures are reached. Near −39° C., critical size becomes comparable to the size of random aggregations of water molecules, and the probability for nucleation rises sharply. Minus 39° C. is considered to be the limit for supercooling.

Once a crystal nucleus has developed and crystal growth continues, the ice formed is composed of virtually pure water, regardless of the solution from which it is formed. The growing ice crystal removes the water from the solution, leaving behind an increasing concentration of solutes. However, not all water in a solution will freeze. Water has many complex functions in the biological milieu and is closely associated or chemically bound with many organic compounds. Loosely bound water will not crystallize until the temperature has fallen several degrees below the freezing point. Tightly bound water, which constitutes 8 to 10 per cent of the water content of mammalian tissue, never freezes.

Let us now consider a typical mammalian tissue, in which we shall observe the following sequence of events as temperature is lowered slightly below normal. The first effects to be noted are physiological in nature, represent the tissues' or the body's reaction to temperature reduction in the normal physiological range, and do not concern us here. However, the first axiom cited above has already come into play; reaction rates are reduced and metabolism and oxygen consumption is lowered. At temperatures near the freezing point many physiological activities have ceased altogether, and one is already entering the range which, although limited in duration, might be loosely called suspended animation. Many mammals can survive up to 24 hours in complete cardiac and respiratory arrest at 0° C. Isolated tissues can survive still longer. Clearly, if further temperature reduction were possible, a further depression of reaction rates would result, and more prolonged suspended animation could be attained. However, at slightly below 0° C. water begins to separate from the tissue solution as ice.

At this point we make another interesting observation. In animal tissues and cell suspensions ice formation is seen only outside the cells at ordinary rates of freezing. At this point the crystals grow in the extracellular spaces, withdrawing water from both within and without the cell until all freezable water has been removed from the tissue solution. The cells collapse between the large crystals, which occupy nearly 70 per cent of the tissue volume. One would certainly be justified, on observing such massive histological disruption, in predicting that this would incur irreversible damage on a mechanical basis. Much to our surprise, however, we find that the ear of a rabbit or the leg of a dog, for example, can be frozen briefly to temperatures at which freezing is unquestionably complete and yet, following thawing, return to functional recovery. In this way we are forced to the conclusion that the formation of ice crystals in tissue is not necessarily lethal. Apparently, the flexible cell can collapse passively without injury to its internal structure or membrane and, with the release of water from the ice crystals on thawing, the cells reimbibe the water, normal cell turgor is re-established, and function recovers. Such recovery is possible only if the exposure to freezing has been brief, that is, a matter of minutes or even of seconds. Prolonged exposure results in irreversible injury, and for this we must look further than the simple mechanical explanation.

When ice forms, water is withdrawn from solution. This withdrawal results in a concentration of the solutes. When all freezable water has been removed, a saturated solution of all solutes has been achieved. Freezing is thus nothing more nor less than dehydration. Clearly, such a situation hardly provides a satisfactory medium for the complex and sensitive constituents of the cell. Lovelock,[4] in studies on the red cell, has shown that is is the concentration of salts that is primarily responsible for injury, and that the lipoproteins appear to be the most sensitive cell constituents. In general, then, it may be said that tissue injury from freezing is primarily the result of the high salt and substrate concentrations produced by the removal of water to form ice. The ice crystal itself becomes an inert foreign body sequestered in the tissue matrix. Unfortunately for the simplified theory, it is becoming increasingly apparent that the salt denaturation theory does not account for everything. Spermatozoa, for example, are destroyed by freezing and thawing, yet an identical freezing, followed by drying and reconstitution, can result in good survival. Thus we may also postulate that, during freezing, the stage may be set for an injury that is actually consummated during thawing. This phenomenon could conceivably be associated with the release of pure water from the ice crystals and be influenced by the size and distribution of the ice crystals. However, regardless of the mechanism of injury, we have every reason to believe that, if the deleterious effects of ice formation can be prevented, living cells can be carried down in temperature without injury.

In summary, the simplified theory of freezing injury proposes that injury from the alteration of reaction rates at reduced temperature is easily avoidable, since a considerable amount of time is required for the effects of metabolic disruption to become irreversible. The theory further proposes that, when freezing is slow and ice crystals are extracellular, flexible animal cells can collapse passively during dehydration without suffering mechanical injury. This theory, of course, will not apply necessarily to more rigid tissues such as plant cells. It is presumed that, where mechanical injury is not a factor, cell death results from the denaturing effect of high salt and solute concentrations attending the dehydration. Having defined in such simplicity the mechanism of injury, we can now consider ways and means of avoiding this injury in order to attain true suspended animation at very low temperatures.

Based on the foregoing analysis, attempts to prevent injury to mammalian tissues during freezing must be aimed at preventing either the dehydration or the effects of dehydration when water is removed to form ice. Let us consider first the possibility of accepting the dehydration of freezing but attempting to avoid its effects. Since the injury resulting from solute concentration is a chemical process requiring the passage of time, it might be possible to reach a low temperature at which this adverse reaction could not proceed and to reach it before significant damage had been done. The notion of ultrarapid freezing is well known, and Luyet and Gehenio[2] have shown it to be feasible in a number of special cases. Unfortunately, however, rapid freezing does not appear to work for the majority of animal tissues. Among mammalian cells erythrocytes appear to be the exception. These cells can be frozen with good recovery when both the freezing and the thawing are carried out rapidly. Contrary to popular opinion, however, there have been no reports as yet of the survival of more than occasional cells of other mammalian tissues following rapid freezing. A possible explanation for this lies in the observation that very rapid freezing rates produce ice crystals inside the cells rather than exclusively outside them, and mechanical injury may be the primary cause of cell death in this case. The relatively small red cell may survive rapid freezing either because there is less probability for intracellular crystal formation or because its lack of internal structure enables it to tolerate this insult. In any case we must abandon for the time being the rapid freezing approach which, either because of intracellular crystal growth or some other undetermined mechanism, simply does not work.

If we cannot avoid the effects of solute concentration by speeding past them, the next alternative is to reduce the concentration itself. Roughly 90 per cent of the water in a cell is freezable. Most mammalian tissues appear to tolerate the removal of as much as 75 per cent of their water before suffering rapid irreversible change. This means that somewhere in the vicinity of 15 per cent of the cell water must be prevented from freezing if rapid injury is to be avoided. Here, again, one might suggest cooling so rapidly that there is insufficient time for ice crystallization to go to completion. However, the ease with which water crystallizes, the large heat of fusion to be removed, and the inherent slowness of heat conduction conspire to make this approach wholely impracticable except, perhaps, for specimens whose total dimensions are microscopic. In any case, we are still up against the observation that, in practice, rapid freezing does not work.

There is another way, however, to reduce the percentage of water that freezes. As observed previously, water bound or tightly associated with another molecule will not freeze. Consequently, the introduction of a material that will restrain a substantial amount of water and that has simultaneously the attributes of being nontoxic and of diffusing readily through cell membranes might solve the problem. This logical analysis is wholly ex post facto. Several years before biological freezing was described by this basically simple picture, such a protective substance was in fact discovered.

In 1949 Polge, Smith, and Parkes[3] of the National Institute for Medical Research in London, England, discovered that glycerin would protect bull spermatozoa from injury by freezing and obtained successful insemination of cattle from semen that had been frozen in 5 to 10 per cent glycerin. One mole of glycerin ties up 3 moles of water. Many other glycols and many sugars are also effective water binders, but glycerin still remains the additive of choice because of its relative nontoxicity in moderate concentrations and the relative ease with which it passes through most cell membranes. Virtually every tissue of the body, including nerve and ganglia, has been successfully frozen and revived when protected with from 5 to 15 per cent glycerin. This amount of glycerin does not, of course, prevent all ice from forming, but it does serve to reduce the concentration of solutes to a tolerable degree. Speed of freezing is no longer a matter of primary concern. In fact, relatively slow freezing is desirable to provide adequate time for the relocation of permeable constituents as the cellular fluid is concentrated by crystal growth. A freezing rate of the order of 1° C. temperature fall per minute appears to be the most universally satisfactory. Temperatures of storage must be quite low and, for long-term preservation of the order of years, temperatures in the vicinity of −100° C. are necessary. Thawing is not particularly demanding, although it should not be excessively prolonged. The removal of the glycerin following thawing is usually necessary. Lest I give a false impression, glycerin is not without its disadvantages since, as a strong hydrogen binder, it becomes toxic in higher concentrations. Even in low concentrations it may exhibit deleterious effects over a prolonged period of time. The diffusion of glycerin into large masses of tissue is slow, and its removal is even more difficult because of the danger of cell rupture on a simple osmotic basis. However, this technique is at the moment the most universally applicable procedure that will permit the freezing and recovery of viable animal tissues.

Since one or two of the papers that follow deal with rapid freezing, it appears advisable to devote some space to this subject to forestall the confusion that might otherwise result. Rapid freezing is a rather vague term describing freezing that takes place in a matter of seconds rather than minutes. It has also been defined as a rate that produces intracellular ice crystals. Such freezing, as pointed out earlier, is not generally successful for the preservation of viability. However, human red cells in whole blood can be frozen rapidly in liquid nitrogen, with the recovery of about 80 per cent of the cells intact.[5] Small droplets of blood roughly 0.5 mm. in diameter reach liquid nitrogen temperature within about 2 sec. The presence or absence of intracellular ice crystals has not, to my knowledge, been investigated. Since the aim of the rapid freezing procedure is to pass as rapidly as possible through a temperature range where the rate of denaturation is high, thawing must also be carried out with extreme rapidity. The addition of certain organic compounds such as glucose, lactose, and, interestingly enough, the plasma extenders dextran and polyvinylpyrrolidone, can markedly improve the recovery of red cells. In our experience, the addition of 7 per cent glucose permits red cell recoveries as high as 98 per cent, with nearly 90 per cent survival in the circulation. It is here that a word of caution is in order. Additives such as the glycols and the sugars, which are effective protective agents during slow freezing, are not necessarily effective during rapid freezing. Conversely, the compounds that improve recovery of red cells during rapid freezing, specifically those that do not penetrate the cell membrane, are completely ineffectual for protection during slow freezing. In short, the mechanism of injury and the mechanism of additive protection during slow freezing are probably substantially different from those of rapid freezing. Unfortunately, the terms freezing injury, additive, and protective agent are applicable to both cases, and one must bear this in mind lest misunderstandings result. Protective agents that are effective in slow freezing presumably operate by binding water and reducing the degree of concentration. In order to be effective, they must pass through the cell membrane. The mode of action of protective agents during rapid freezing remains obscure, possibly having something to do with an alteration of crystallization velocity.

In summary, then, we can in general find no evidence of any direct deleterious action of reduced temperature. The metabolic imbalance created by low temperatures above freezing is reversible if not too extensive and is of no concern to us here. All of the obstacles to the preservation of life at low temperatures are apparently involved in the crystallization of ice. For this reason the study of freezing in biology, as evidenced by the papers to follow, is immediately concerned with ice crystal growth and its direct consequences. Most of our current understanding of freezing injury to cells is the result of analyses of fortuitously discovered means of avoiding this injury. The next step, we hope, will be the development of a still better understanding of the mechanism of freezing injury. This knowledge will, in turn, enable a further extension of low-temperature applications to more sensitive or more complex organisms. We look forward to the use of suspended animation at low temperature as a valuable research tool, permitting the study of transient phenomena through the preservation of time-labile biological characteristics.

The future of cryobiology is exciting, permitting as it does the attainment of indefinitely suspended animation, an unnatural state that paradoxically can augment our understanding of natural processes. Whether this process is becoming uncomfortably unnatural or, indeed, whether anything that can be done can be called unnatural, is a purely academic question. Regardless of one's opinions, suspended animation in isolated cells and tissues is in fact a reality. For the first time in his role as manipulator of the environment, man has altered the time dimension of his existence, since, for the red cell, the sperm, or the kidney suspended at low temperature, time does in fact stand still. We must look for comfort in the hope that time, although interruptable at low temperature, will prove at least to be irreversible.

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THERMAL ANALYSIS OF EUTECTICS IN FREEZING SOLUTIONS

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The transformations of complex systems according to temperature changes are generally very difficult to analyze mathematically, and a precise theoretical study can be made only for simple systems with a limited number of components.

In the case of biological materials the problem is an acute one because of the high level of organization of the protoplasm of the tissue cells. At any rate, one component, water, seems to play a very important role, and most of the thermodynamic properties of the living matter are related to the particular behavior of water itself.

General Considerations on the Freezing of Diluted Aqueous Systems

Ice crystals are generally formed when highly hydrated systems are cooled to temperatures below 0° C. In some very special cases where high cooling velocities can be realized there is no ice crystal formation but a direct hardening of the system, which then assumes a glasslike structure. I shall not consider this particular aspect of the freezing of biological material. The number of germinative centers, the distribution, growth, and subsequent evolution of the crystalline nuclei depend upon several different internal and external factors. The internal factors derive from the very nature of the system itself and include the water content, structure and shape of the specimen, and the nature and concentration of solutes and dispersed phases. The external factors include absolute temperature, specific heat of the cold source, and the nature of the thermic contact between the cooled body and the refrigerating system. The cooling curve and the texture of the frozen specimen result from the interaction of these different factors and vary widely from one example to another.

However, it is possible to make some general remarks on the dynamics of freezing in biological material. First, I feel that it is necessary to formulate some physical definitions in order to settle the problem. Then we shall see how it is possible to extrapolate from them to complex systems, especially biological material.

To study the transformations of a simple aqueous system, such as a solution of one mineral salt in distilled water, it is necessary to establish its equilibrium diagram (FIGURE 1). The different states of the system (gaseous, liquid, solid, and hypercritical) are represented on a three-dimensional diagram where pressure, temperature, and concentration can be plotted separately. As pressure is most often constant, the diagram can be simplified and reduced to an isobar section of the equilibrium surfaces, the only two variables being temperature and concentration. Usually the system will behave as an isodimorphous binary where two different types of crystals can be formed (Pascal, 1953).

If we look at the thermodynamic diagram, we see that there are two different possibilities: the curves forming the liquidus can join in a transition point or in a eutectic point. In the first instance, for a certain range of concentrations (FIGURE 2), the crystallization leads to an invariance phenomenon, and the temperature remains stable during the whole length of the peritectic decomposition reaction. In the second case, which is the most frequent for diluted solutions, the liquidus curves join in a eutectic point (FIGURE 3), and the crystallization leads first to the formation of ice crystals. This process produces a progressive concentration of the solute, and the figurative point follows the curve down to the eutectic point *E*. The system then becomes invariant under the fixed pressure of the experiment, and the phase composition remains constant. This condition imposed upon the liquid can be fulfilled only if crystals from both constituents, water and mineral salt, are formed simultaneously, giving an intermingled conglomerate, the composition of which is identical with the one of the eutectic liquid *E*. The result will be a solid containing ice crystals surrounded by the solid eutectic mixture. If the initial solution has the exact composition of the eutectic liquid, it will freeze as a pure substance; that is why it has been called the cryohydrate. Any frozen diluted solution can then be represented as a conglomerate of ice and cryohydrate crystals.

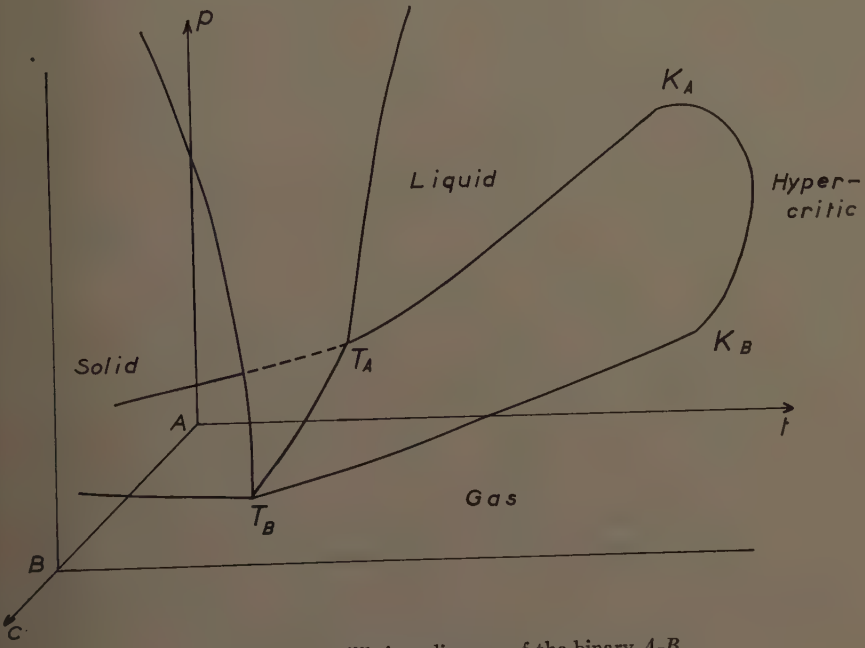

FIGURE 1. Equilibrium diagram of the binary *A*-*B*.

For most mineral salts a eutectic temperature can be found and measured accurately. However, in some instances the crystallization of the eutectic mixture can be delayed, and metastable equilibria can be reached. It is then necessary, as we shall see, to cool the system in order to induce complete crystallization previous to subsequent rewarming.

From the preceding observations we can derive some important characteristics of the eutectic points:

For most aqueous solutions there exists a certain temperature (the eutectic temperature) at which complete crystallization is achieved and further cooling does not introduce new structural changes. This temperature is characteristic of the binary, which is cooled and independent of its initial composition.

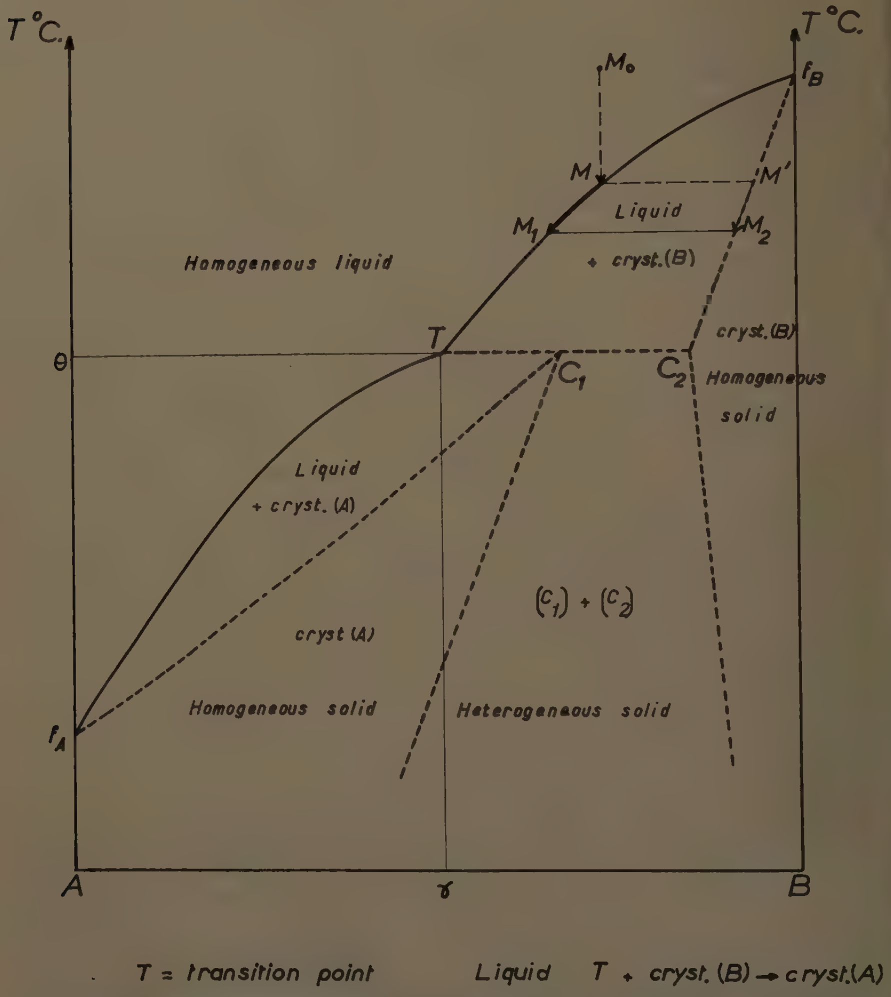

FIGURE 2. Thermodynamic diagram of the binary A-B in the case where the liquidus curves join in a transition point T. M_0 shows the evolution of a solution that is highly concentrated in B. When M_1 and M_2 reach the temperature θ the system becomes invariant during the entire length of the peritectic decomposition reaction.

The frozen solution is made of ice crystals surrounded by solid eutectic mixture, and the proportions of both depend upon the initial salt concentration.

The phenomenon is reversible and, when the system is rewarmed, the eutectic mixture melts if the temperature is raised above the eutectic point. Actually, when the exact eutectic temperature is reached, the system becomes invariant as long as some frozen cryohydrate remains, and the temperature can be recorded accurately. It is because of the eventuality of supercooling phenomena that the precise determination of the eutectic temperature is generally done in the course of rewarming. When the eutectic mixture has disappeared, ice

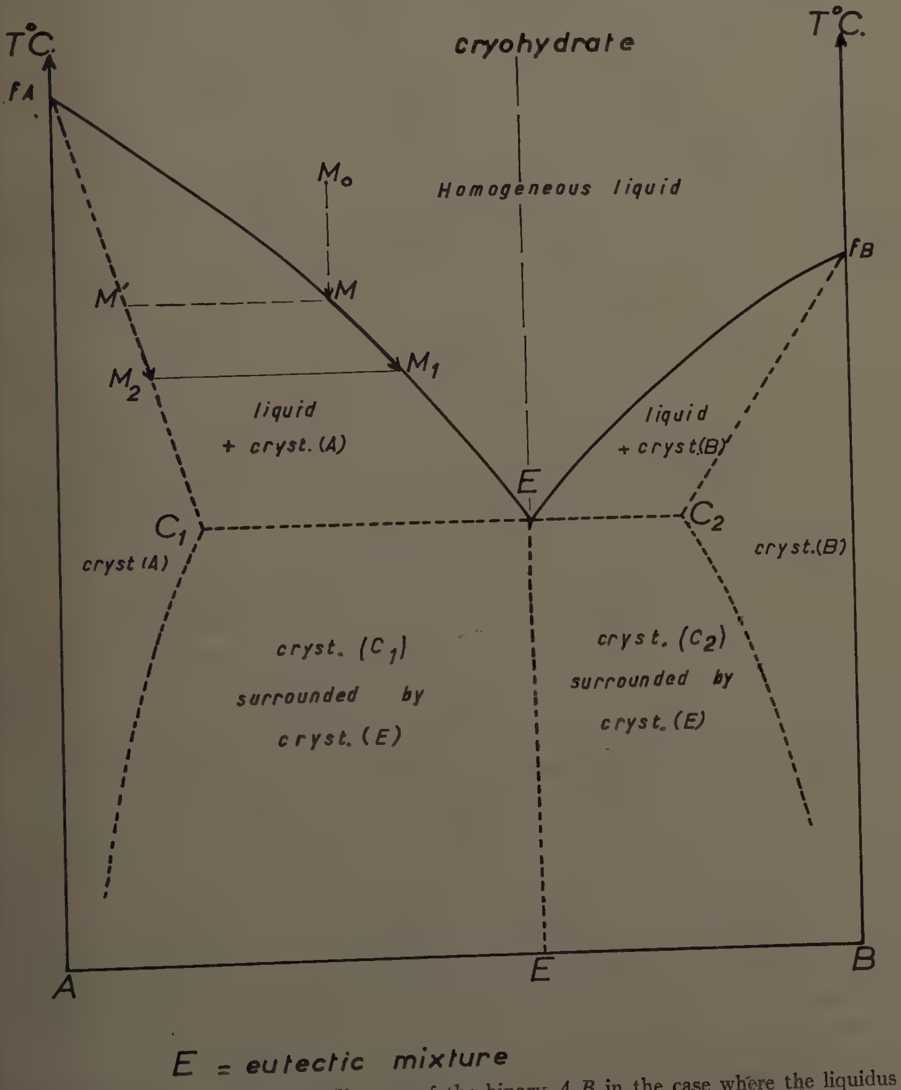

FIGURE 3. Thermodynamic diagram of the binary *A-B* in the case where the liquidus curves meet in a eutectic point *E*. M_0 shows the evolution of a solution highly concentrated in *A*.

begins to thaw if rewarming is continued, and the amount of liquid increases as more and more ice crystals melt and feed the interstitial fluids, the last crystal of ice disappearing at a temperature corresponding to the normal cryoscopic point of the initial diluted solution.

Is it now possible to extend these conclusions to more complicated systems and especially to complex salt solutions and biological material?

We may say, first, that eutectic phenomena, as just described, do not exist in complex solutions, but at any rate it is possible to extend the definition with some precautions. In accordance with usage established in the literature, I shall extrapolate and speak of eutectic phenomena in biological material. Accordingly, a eutectic temperature or a eutectic zone is defined as the maximum temperature at which total crystallization of the material can be achieved. Above the eutectic temperature the system still contains liquid phases, and below the eutectic point everything has turned to the solid form.

My intention in this short review is not to deal with all the details of the eutectic crystallization in biological products, but to limit the discussion to a given number of examples of rising complexity. First, let us determine what are the methods that can be used for the determination of eutectics and then describe some of the principal results.

Structure of the Frozen Biological Systems as Studied by the Thermal Analysis Method

EXPERIMENTAL TECHNIQUES

In order to study eutectic phenomena in solutions, I have been using different methods that are briefly summarized below.

Crystallization Velocity

Most biological materials are susceptible to being supercooled to a rather large extent, and crystallization occurs quite abruptly. The velocity of growth of the ice crystals can be measured accurately. For that purpose, I have been using a device similar to that of Lusena (1955). The solution is kept inside a capillary tube that is cooled to the desired temperature. It is then seeded at one end with an ice crystal, and the velocity of the crystallization process is determined. So as to be able to follow more easily the movement of the crystallization edge, the observation is done in polarized light, the polarizing and analysis directions being situated at an angle of 45° to the axis of the capillary tube (Rey, 1959*a*).

Thermal Analysis

In order to follow very accurately the thermodynamic behavior of the frozen solutions, my associates and I have developed in our laboratory the thermal analysis method. This technique has long been used in metallurgy and was recently applied to mineral chemistry by G. Vuillard (1957). The principle of the method is to record the temperature curve of the specimen when it is cooled or rewarmed at a constant rate. As any structural change is generally linked to a thermal effect, the diagram will be modified, and the exothermic or endothermic phenomena will be easily seen. For instance, any freezing or melting of a eutectic mixture will be indicated by an alteration of the slope of the curve, as it is an invariance state.

To increase the sensitivity of this direct thermal analysis technique we have primarily been using differential thermal analysis (Vuillard, 1957). The temperature of the specimen is then continuously compared to the temperature of a reference system placed in the same conditions, and the difference is plotted against the absolute value of the temperature of the specimen (Rey, 1958*a*).

Because of the possibility of supercooling phenomena we do the thermal analysis in the course of rewarming. The solution is first cooled to liquid-nitrogen temperature at various speeds (from 2 to 20 min.) and then rewarmed regularly to the thawing point. During that process direct and differential thermal curves are automatically recorded, and the variations in resistivity can be followed.

A special type of experimental cell is used to contain the solutions. Made of stainless steel, the metallic block has 4 holes into which the solutions can be placed. Highly sensitive thermocouples, held by the upper part of the cell, can be immersed in the solutions (FIGURE 4).

Electric Conductivity

R. I. N. Greaves (1954) was one of the first investigators to use electric conductivity to follow the terminal phase of the crystallization process, and our technique is very similar. We measure the electric resistivity of the frozen solution in low-frequency current (1000 cycles), and we make a logarithmic plot against temperatures. As long as some liquid is still present inside the specimen the electric current will flow throughout the frozen mass without too much difficulty. When everything is solidified the resistivity becomes very high, and this sudden increase of resistance is the indication of the crossing of the eutectic border.

EUTECTIC CRYSTALLIZATION IN SALINE SOLUTIONS

Binary System of Sodium Chloride and Water

In a first set of experiments solutions of sodium chloride in water were studied. This solution is rather easy to handle, and its thermodynamic diagram is very well known (FIGURE 5). A eutectic point can be found at −21.6° C., and the eutectic mixture corresponds to 23.6 per cent of anhydrous NaCl.

Thermal analysis. Solutions of different initial concentrations have been examined; the results are shown in FIGURE 6.

There is a very precise eutectic thawing at −21.6° C. The thermal effect shows a maximum for that temperature, and the importance of the deviation is a function of the amount of eutectic present in the solution. As shown by Vuillard (1957), the segment *AB* corresponds to the duration of the eutectic thawing, and the area *ABC* is proportional to the amount of mixture that disappears. This may be shown by comparing the curves corresponding to solutions of different initial concentrations (FIGURE 7). The melting of ice crystals follows eutectic thawing immediately and becomes important from −16.5° C.

In some cases a slow exothermic phenomenon occurs before the eutectic temperature is reached, beginning between −90° and −80° C. (FIGURE 8). I believe that this may indicate a partial recrystallization of the solution in the course of rewarming that can be correlated to the migratory recrystallization

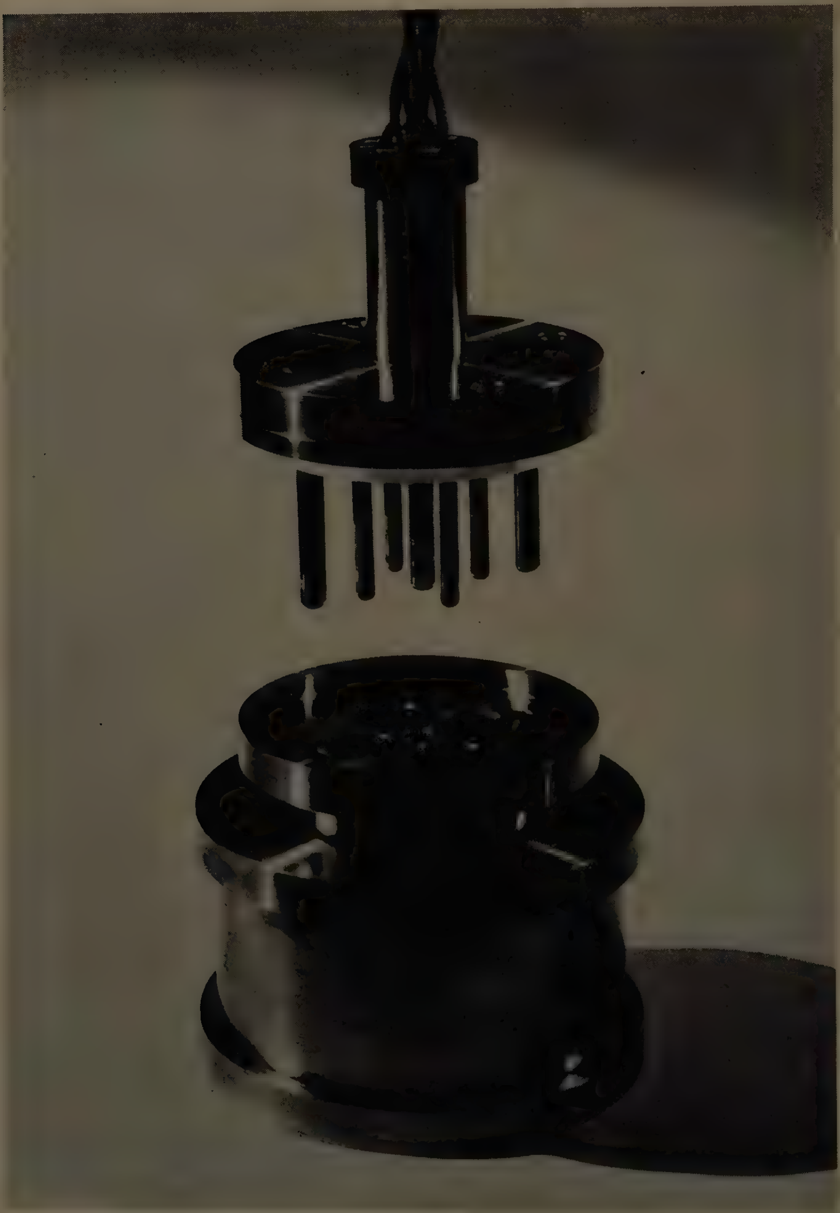

FIGURE 4. Experimental cell used in thermal analysis measurements.

described by Meryman (1955). At any rate, this is not always clearly seen and must correspond to a special freezing process.

Resistivity measurement. Electric resistance measurements have been done during cooling and rewarming; the results are shown in FIGURE 9. From the curves it is possible to draw the following conclusions.

There is a marked discrepancy between the freezing and the thawing curves; in particular, high electric resistances are reached at much lower temperatures when cooling than in the course of rewarming. This indicates that there is a constant tendency of the eutectic solution to supercool, and this is one reason why it is not advisable to determine eutectic points on freezing curves.

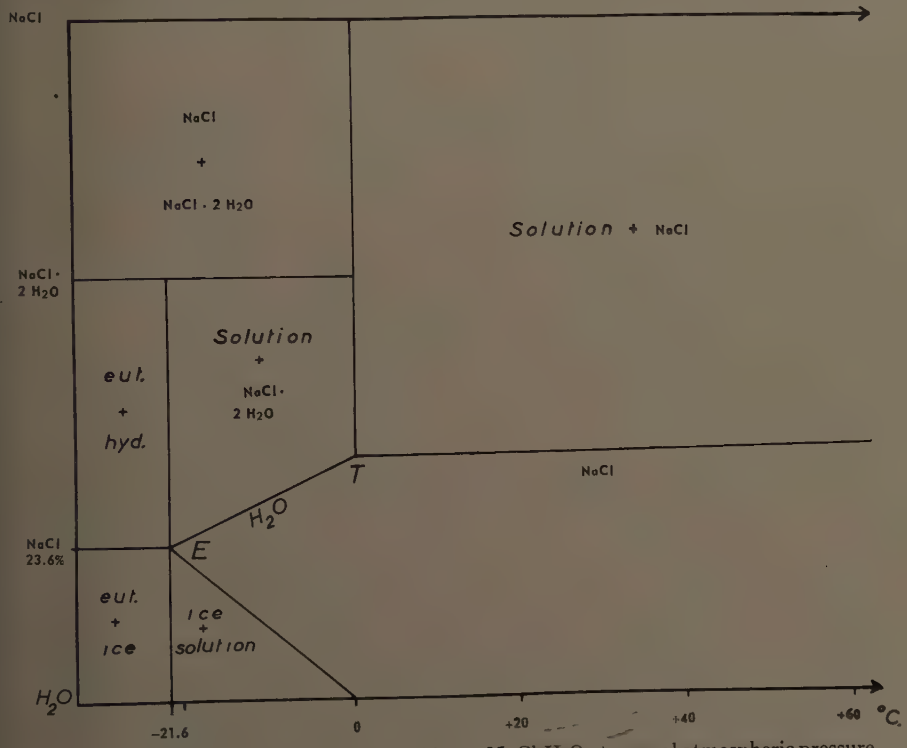

FIGURE 5. Equilibrium diagram of the binary NaCl-H_2O at normal atmospheric pressure.

On the other hand the eutectic thawing at −21.6° C. is clearly seen during rewarming, as it corresponds to a tenfold decrease of the resistivity.

There is a slow and progressive change of the electric resistance starting in the neighborhood of −80° C. and becoming more important after −45° to −50° C. We think that it corresponds to structural changes of the solution, which can be very slow and may not yield thermal effects. As a hypothesis, we suppose that it can be attributed to recrystallization movements of the same type as those that sometimes are seen on the differential thermal diagrams (FIGURE 8).

Microscopic observations. The direct observation of the crystallization and thawing of the eutectic mixture has been made possible by the use of the freezing microscope devised in our laboratory (Rey, 1957). We obtained confirmation of the fact that the eutectic fluid was very susceptible to supercooling (as low as −35° C. and even −40° C.) when cooling is progressive (FIGURE 10). It can then freeze abruptly (FIGURE 11) either by lowering the temperature or

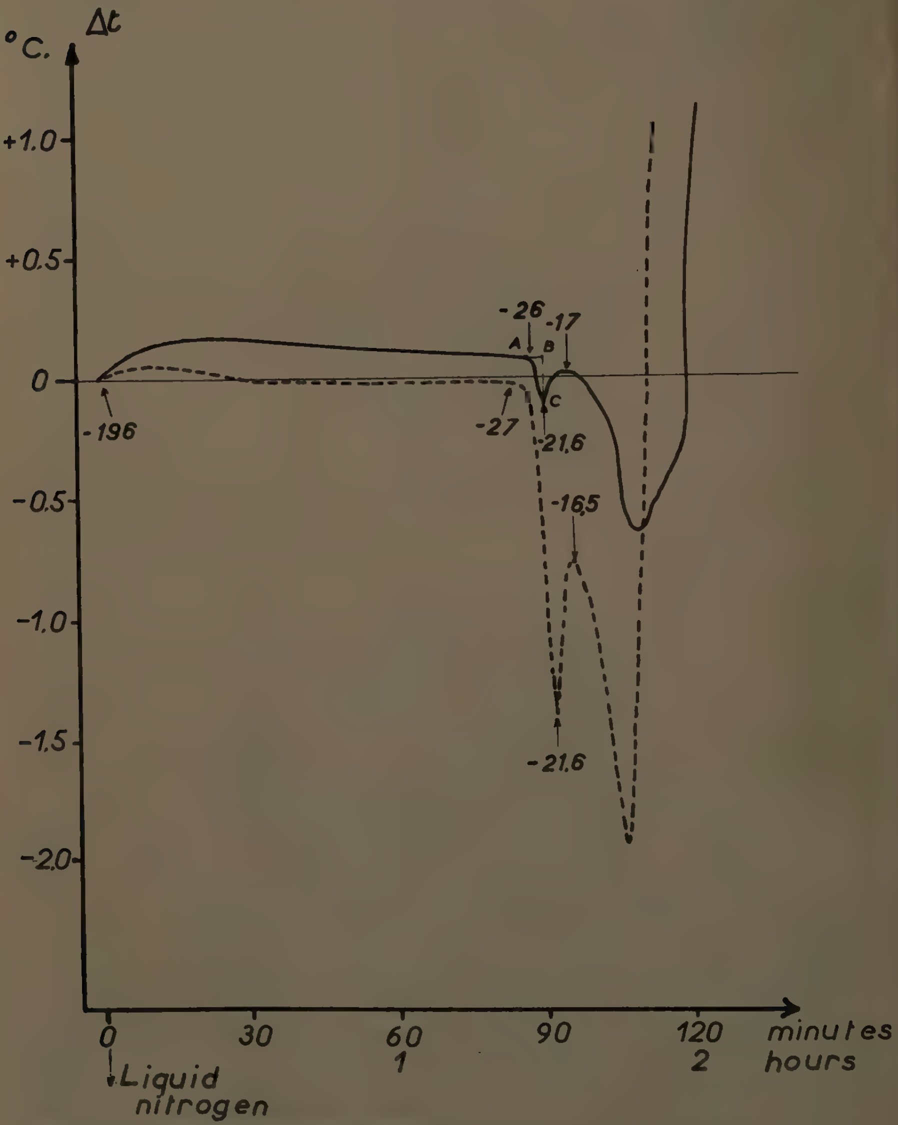

Figure 6. Differential thermal analysis diagram of the system NaCl-H_2O. *Solid line,* 10 parts per 1000 solution of NaCl in distilled water; *broken line,* 100 parts per 1000 of NaCl in distilled water.

raising it again and allowing it to remain stable at 2° or 3° C. below the eutectic point. At any rate, when frozen, the eutectic always melts instantaneously when the temperature reaches −21.6° C. The dissociation of the frozen eutectic mixture is immediate, and the resulting crystals of ice and of the hydrate $NaCl \cdot 2H_2O$ melt rapidly. If the initial concentration is high, so that there is a great amount of eutectic, this can produce a complete dislocation of the frozen mass, and the ice crystals can move at random in the surrounding hyperconcentrated solution, which has the exact composition of the eutectic fluid. In this case the size of ice crystals begins to diminish up to the final thawing if rewarming continues.

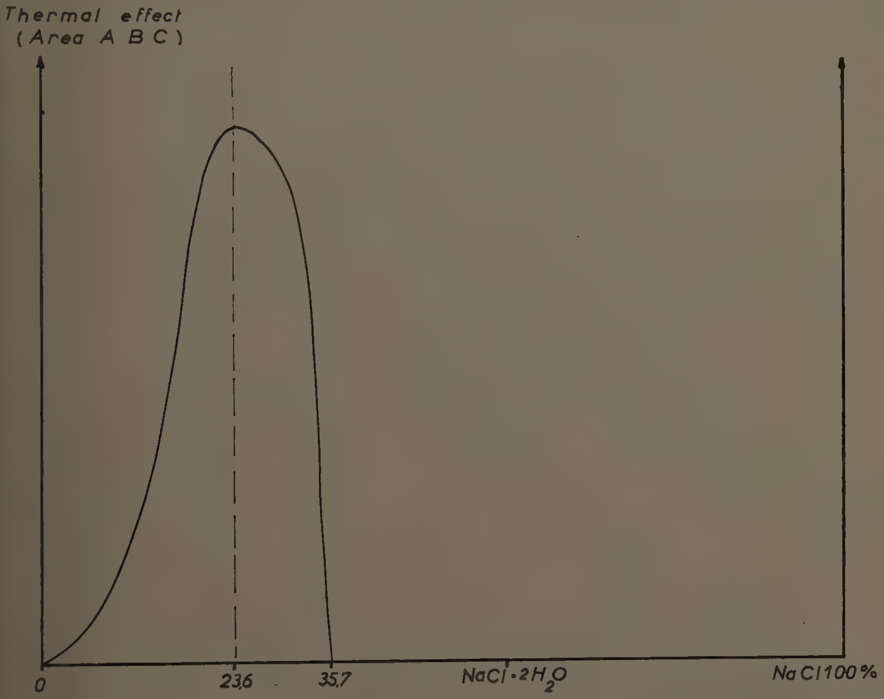

FIGURE 7. Importance of the thermal effect bound to the eutectic melting in aqueous solutions of sodium chloride according to the concentration of NaCl.

Influence of agar-agar on the crystallization process of a sodium chloride solution. A second set of experiments was initiated to determine how the crystallization would take place in a gel instead of a liquid; for this purpose agar-agar was added to the aqueous solution of sodium chloride. FIGURE 12 shows the differential thermal analysis curves corresponding to a 1 per cent solution of agar-agar (Bacto-agar Difco) containing 10 parts per 1000 and 100 parts per 1000 of sodium chloride, which gives a relatively rigid gel. Those diagrams present the following characteristics: the exact temperature of the eutectic point is not modified, and remains equal to −21.6° C.; the amplitude of the thermal phenomenon is slightly reduced; and, for a higher proportion of agar-

agar (that is, 5 per cent), the eutectic peak is very small and its position shifts towards lower temperatures (−24° C.). A separate analysis has shown that there was no special thermal phenomenon occurring during the freezing of a 1 per cent solution of agar in distilled water, with the exception of initial water freezing.

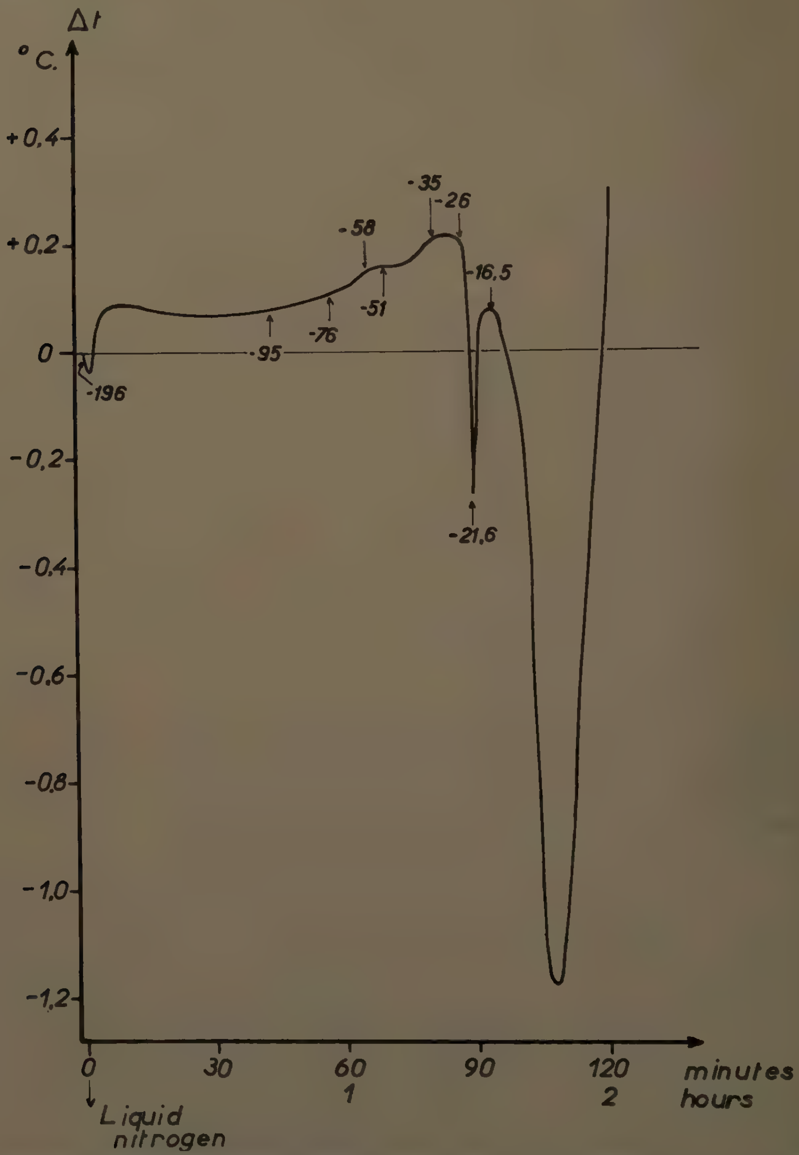

FIGURE 8. Differential thermal analysis diagram of a 10 parts per 1000 solution of NaCl in distilled water in the case in which an exothermic phenomenon can be found prior to the eutectic thawing.

Other Simple Saline Solutions

Binary system of potassium chloride and water. Similar phenomena can be observed for potassium chloride solutions. The eutectic temperature is higher: −11.5° C. The addition of agar-agar has the same effect. For a 5 per cent solution of agar-agar in a 10 parts per thousand solution of KCl, the eutectic temperature is situated at −14° C.

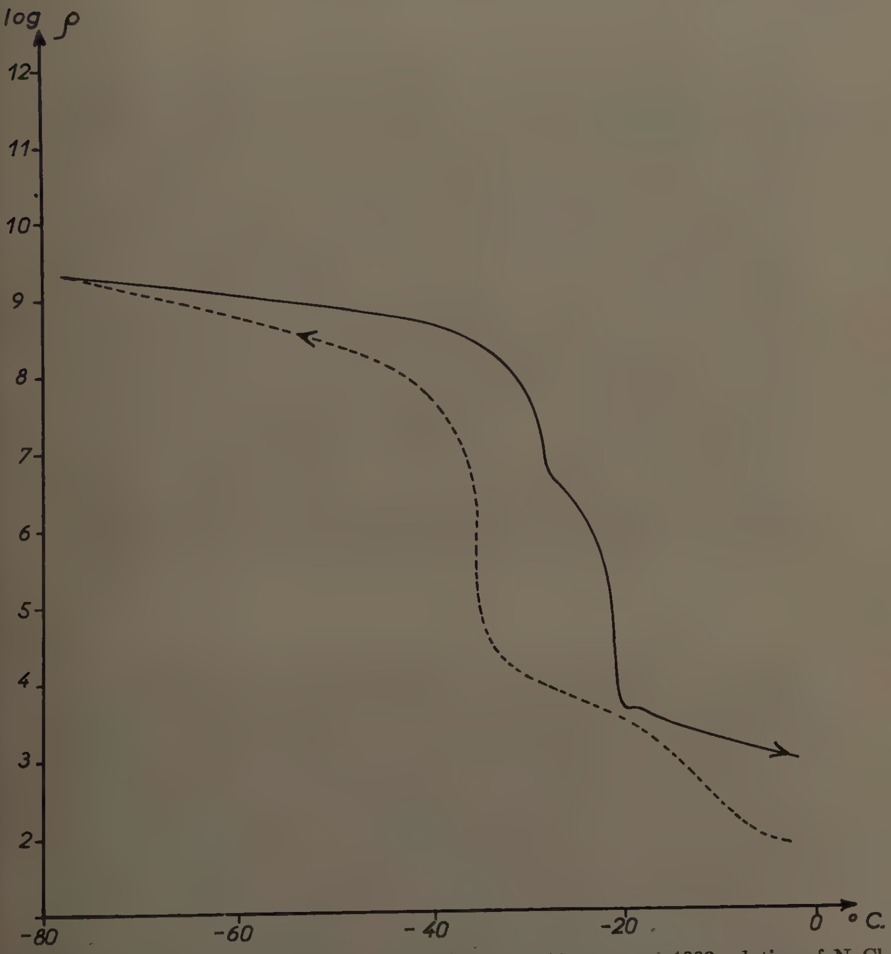

FIGURE 9. Variation of the electric resistance of a 10 parts per 1000 solution of NaCl in distilled water according to low-temperature changes. *Solid line,* measurements taken in the course of rewarming; *broken line,* measurements taken during the cooling process.

Binary system of ammonium chloride and water. The eutectic temperature is −15° C.

Mixed chloride solutions. We have analyzed mixed solutions of chlorides. As a rule, the eutectic temperature is slightly modified, and the eutectic peak is widened and sometimes presents very slight lateral undulations. TABLE 1 presents some numerical data concerning our experimental solutions.

Complex Salt Solutions: Earle's Solution

We have extended our investigation method to more complex solutions and especially to balanced salt solutions so as to remain closer to the biological ma-

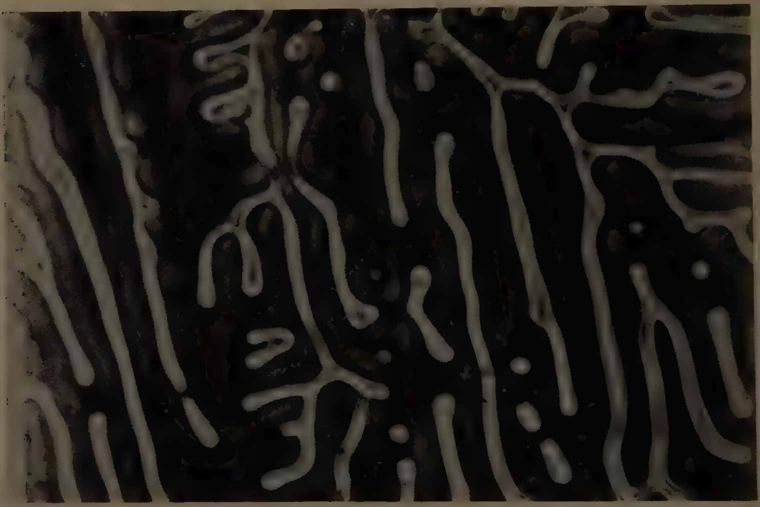

FIGURE 10. Microscopic examination of the crystallization of a solution of NaCl (100 parts per 1000) in distilled water. Supercooled eutectic fluid can be seen within the white channels between the ice crystals. Temperature, −35° C. Phase contrast. ×350.

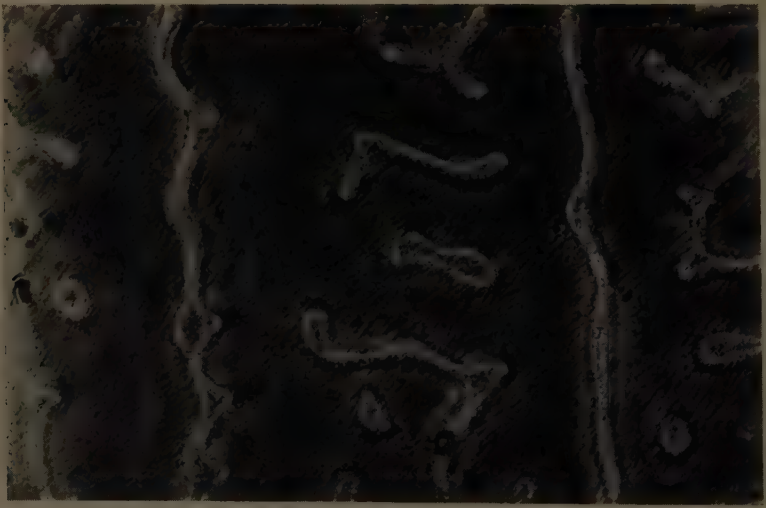

FIGURE 11. Microscopic examination of the crystallization of a solution of NaCl (100 parts per 1000) in distilled water. Eutectic mixture was crystallized out on top and between the ice crystals. Temperature, −22° C. Phase contrast. ×350.

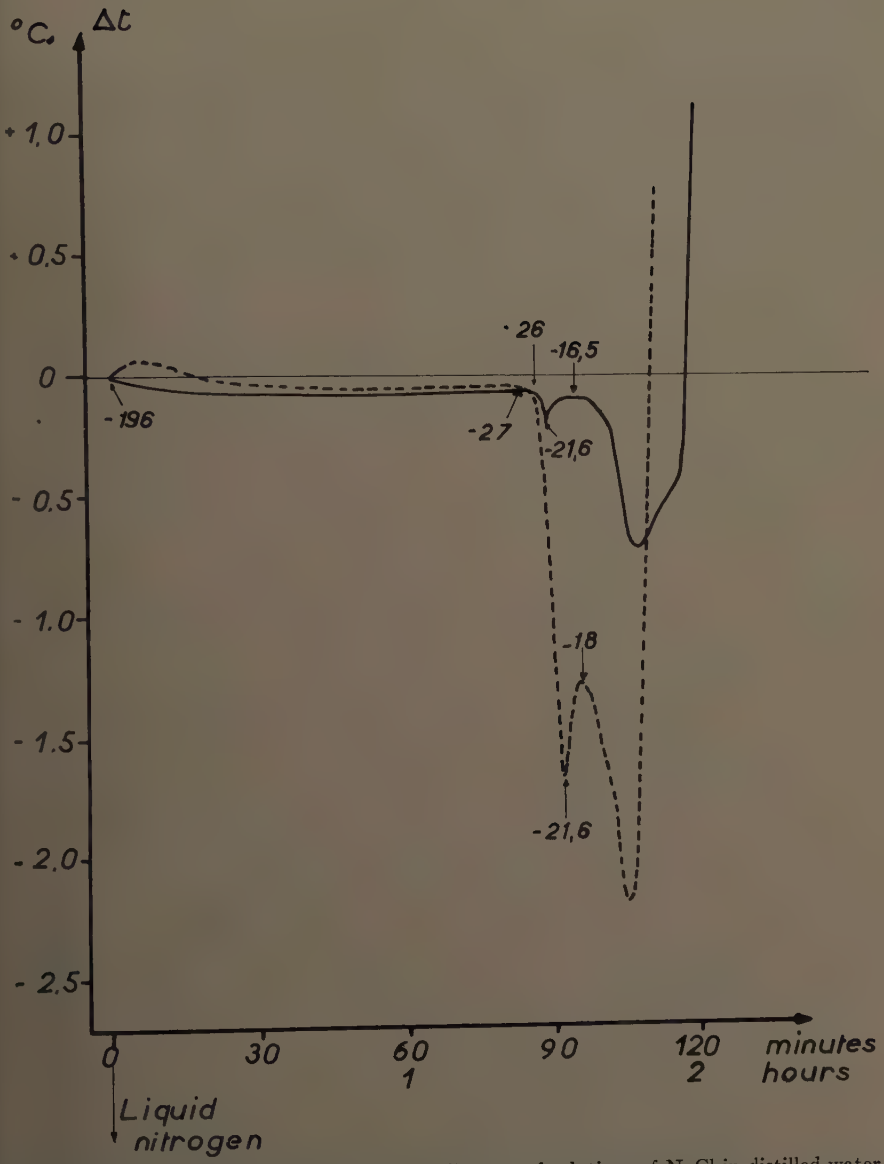

FIGURE 12. Differential thermal analysis diagram of solutions of NaCl in distilled water in the presence of agar-agar. *Solid line*, NaCl (100 parts per 1000) in agar-agar gel (10 parts per 1000) in water; *broken line*, NaCl (100 parts per 1000) in agar-agar gel (10 parts per 1000) in water.

TABLE 1
COMPOSITION OF EXPERIMENTAL SOLUTIONS

Solution (parts per 1000)	Eutectic temperature (°C.)
NaCl (5) + KCl (5)	−23.5
NaCl (5) + NH_4Cl (5)	−24
NaCl (3.3) + KCl (3.3) + NH_4Cl (3.3)	−27.5

terial, at least as far as total salt concentration is concerned. For this reason, we have been working on Earle's solution (TABLE 2).

Thermal analysis. The differential thermal analysis curve (FIGURE 13) indicates a marked endothermic phenomenon with a maximum for −24° C. This can be attributed to a real eutectic thawing because of the presence of a well-defined peak on the diagram. However, that important structural change seems to be preceded by more delicate endothermic effects that can be attributed to the thawing of different phases in small amounts corresponding to heterogeneous saline mixtures.

Resistivity measurements. The electric measurements give confirmation of that hypothesis, as there is no sharp eutectic border (FIGURE 14). The curves show a progressive modification of the resistivity instead of abrupt variations. At any rate, the following conclusions can be drawn from these experiments.

Eutectic thawing is always found at the same temperature in the course of rewarming and actually corresponds to a definite thermodynamic phenomenon.

TABLE 2
COMPOSITION OF EARLE'S SOLUTION

Ingredient	Grams per liter
Sodium chloride	6.80
Potassium chloride	0.40
Calcium chloride	0.20
Magnesium sulfate	0.20
Monosodic sulfate	0.14
Sodium bicarbonate	2.20
Glucose	1.00
Water (twice distilled)	to make 1000 cu. cm.

On the other hand, as has been seen for sodium chloride solution, supercooling occurs during slow freezing; this is emphasized by the discrepancy between the cooling and heating curves.

The hardening of the solution is completed only at rather low temperatures, and it is necessary to go down at least to −35° C. to have a resistivity of 1 Mω/cm.

The following conclusions may be drawn from the above experiments on the behavior of aqueous saline solutions at low temperatures.

The texture and fine structure of a frozen solution depends not only upon temperature, but also upon the history of the freezing process. A solution slowly frozen to −40° C. will differ widely from the same solution first cooled to −80° C. and later rewarmed to −40° C. In other words, there is no definite structure that corresponds to any given temperature. This fact can have very important practical implications, especially in freeze drying. Actually, if one wishes to ascertain that a solution is completely frozen and that no more liquid remains between the ice crystals, it is wise to cool the system first at much lower temperatures than would be necessary if one considered the eutectic temperature values alone. On the other hand, precise eutectic temperatures are difficult to calculate for complex salt solutions. The eutectic temperature of a group of salts cannot be derived from the different eutectic points of the individual components. Accurate experimental measurements must be made and, in most cases, as the composition of the solution becomes more and more complicated, sharp eutectic boundaries are replaced by a eutectic zone where a progressive crystallization of the saline mixtures takes place.

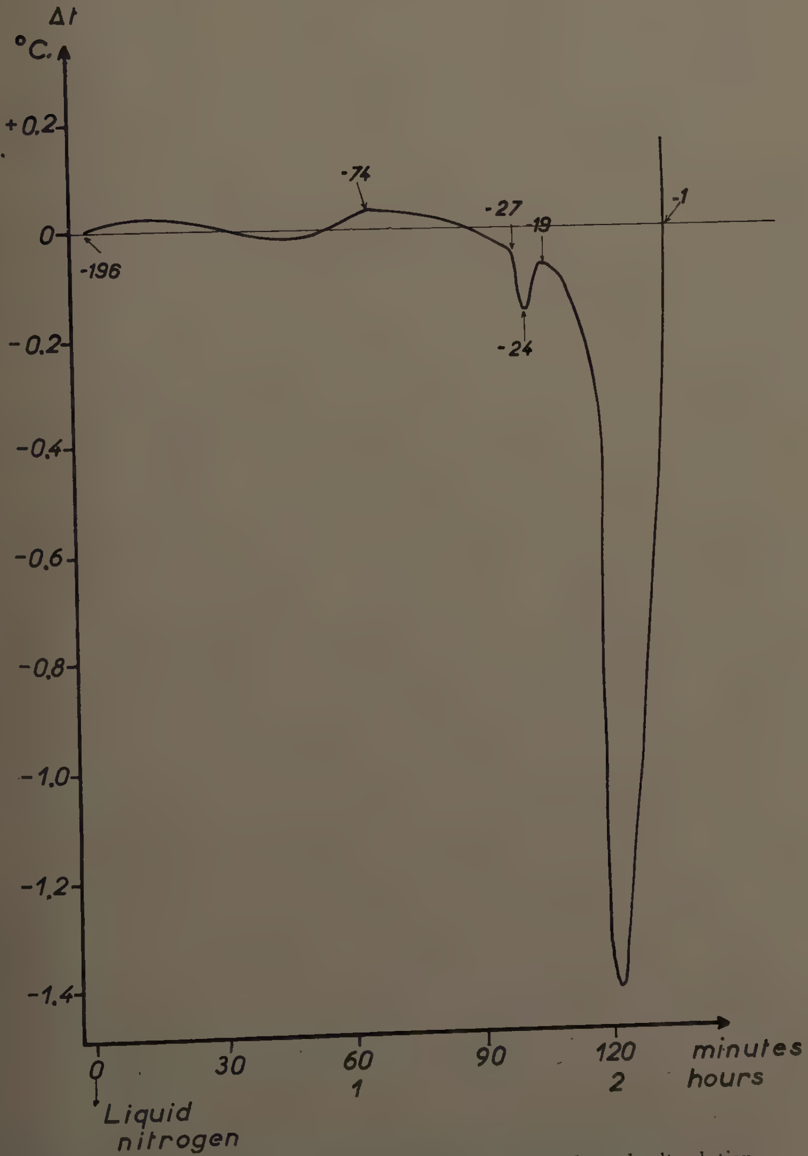

FIGURE 13. Differential thermal analysis of Earle's balanced salt solution.

SOME EUTECTIC PHENOMENA IN BIOLOGICAL PRODUCTS: EXPERIMENTAL STUDIES AND PRACTICAL APPLICATIONS

Lyophilization of Complex Biological Systems: General Remarks

We have seen previously that it was very difficult to conduct precise experimental studies on the low-temperature behavior of complex solutions, especially of biological material. It thus appears that it is nearly impossible to measure accurately a eutectic temperature inside a natural substance, or even to give a correct definition of the eutectic phenomenon in such a complicated system. This is certainly true, but we believe that, with some preliminary precautions, the eutectic phenomena in biological products can be approached in such a way that these studies can have some very important theoretical and practical applications. I shall report briefly some experimental results that must be considered as isolated examples that do not necessarily introduce general rules.

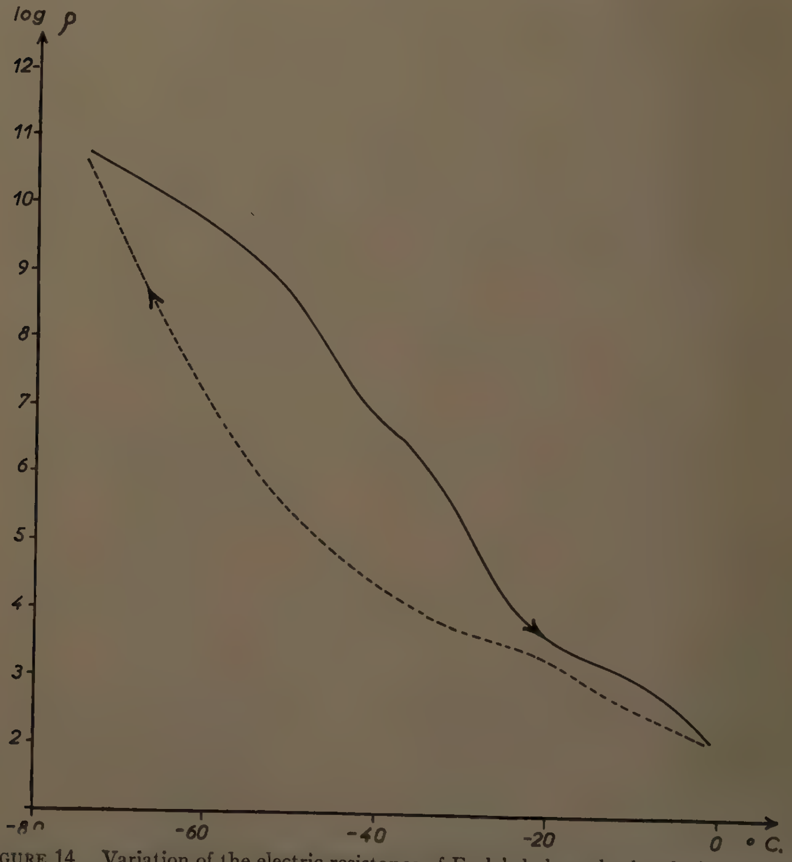

FIGURE 14. Variation of the electric resistance of Earle's balanced salt solution according to low-temperature changes. *Solid line,* measurements taken during rewarming period; *broken line,* measurements taken during cooling period.

Many biological products, especially those of extractive origin, cannot be defined clearly in terms of their chemical composition. The very high complexity of their structure and the huge amount of different components prevent a precise analysis. However, such products can easily be identified by biological assays or by study of their biophysical properties. For instance, both horse serum and chick embryo extract have special biological and physical properties that can be used to check their respective purity and quality. Electrophoresis, ultracentrifugation and sedimentation patterns, *p*H value, and viscosity permit an accurate determination of the main characteristics of horse serum. In the case of embryo extract, *p*H value, viscosity measurements, and especially biological tests such as tissue culture and organ culture give sufficient information on its global composition and activity.

The recent development of global organotherapy and the discovery of the numerous applications of tissue extracts in the clinical field and in the laboratory have made it necessary to find convenient methods of preparing and preserving complex biological substances. Among the techniques that can be used, lyophilization is certainly the most adequate, since it allows a long-term storage of highly alterable products at normal room temperature. This is why more and more natural products have been dried under vacuum after previous freezing. Recently this method has been extended to tissue banking on the one hand, and to food industry and large scale production of such items as frozen and dried meat, fruit juices, and vegetables, on the other. Consequently, the size of the apparatus used has increased considerably, and the economic importance of the lyophilization process grown rapidly.

It can easily be understood that, under those conditions, the quality of the preservation of the dried biological material is of the utmost importance. The products must not only be kept free from subsequent alterations in the course of storage, but they must be treated with great care to avoid denaturation. It has been thought for too long a time that good reconstitution of the dried material was the only criterion of an excellent lyophilization. The aspect of the dry substance, its ability to keep its rehydration power, its easy dissolution, the absence of precipitates, and the maintenance of a normal *p*H value all give very valuable information. However, experiments on more difficult products such as cornea, vaccines, or foodstuffs have shown that the lyophilization process must be worked out very precisely in order to preserve all of the initial properties of the substances. For instance, a cornea must remain perfectly clear after dehydration; a vaccine must keep its entire immunizing power; and a piece of meat must show normal organoleptic properties. It is then necessary to adapt the lyophilization technique to any given particular case.

There is insufficient space to deal here with all of the different aspects of this problem. However, we feel that most of the information necessary to perform a good lyophilization can be derived from laboratory experiments and, principally, from eutectic measurements (Rey *et al.*, 1959).

Actually, one of the most important factors in freeze drying is the regulation of the temperature of the specimen in the course of drying. That temperature must be sufficiently low to prevent any melting of the solution and sufficiently high to allow the lyophilization process to be completed within a reasonable time. An equilibrium between security and productivity must be reached that is always a compromise.

We have found that the freezing period and the actual temperature of the specimen during drying are the most important points of the whole technique, and both are related to the eutectic phenomena. Indeed, it appears that a limited melting of the eutectic mixtures or a softening of the hard structures of the frozen solutions is responsible for the denaturation processes that generally occur in an unsuccessful lyophilization. Of course, if melting is more important, partial thawing and puffing will occur, but these are dramatic morphologic changes that never appear in a normally performed lyophilization. There are many more intermediate cases where the drying seems apparently good, but where a slow and discrete denaturation process has taken place during the operation, resulting in a tremendous decrease in the activity or quality of the product.

In those liminar cases, we feel that a small modification of the prefreezing technique or a few degrees of difference in the temperature of drying would be sufficient to avoid denaturation. Speaking in terms of eutectics, we should say that the knowledge of the exact position of the eutectic zone should help in preventing such incidents. As we have said before, the term eutectic zone is used here only by analogy with what can be found for saline solutions and is used merely for convenience.

To determine the "eutectic requirements" of a natural substance to be frozen and dried we recommend the following procedure.

(1) Precise determination by resistivity measurement of the maximum temperature of complete solidification (T_{cs}), that is, the highest temperature at which it is necessary to cool the product to be sure that supercooling effects are no longer present and that all liquid phases have turned to solid forms.

(2) Exact determination by thermal analysis of the minimum temperature of incipient melting (T_{im}), that is, the lowest temperature at which some endothermic phenomena begin to appear that can be correlated to partial or total melting of eutectic mixtures or related complex structures.

When these elements are known, the lyophilization process can be adjusted in such a way that the substance will be frozen previously at a temperature equal to or below T_{cs} and, in addition, that the temperature of the specimen during active sublimation always remains below T_{im}.

Of course, it is possible to formulate, without any eutectic measurements but with repeated experiments done in different conditions, an adequate technique corresponding to a given product, and this has been done routinely in most lyophilization plants. However, this constitutes a loss of both time and money and does not actually give the certitude that it is the best way to proceed. It can occur that a given product dried at −30° C. could be dried equally well at −20° C., and it is easy to see what are the economic consequences of that small difference when one considers the variation of the time of drying according to the temperature of sublimation.

In many cases, especially for industrial work, the determination of the exact temperature of freezing is not an absolute necessity, as most processors cool their product to the lowest possible temperature that can be achieved with their equipment. This is certainly a good precaution, but it can occur that for some special material this temperature is insufficiently low and that it may be necessary to apply dry ice or liquid nitrogen to help in the prefreezing process. Consequently, it appears that a precise determination of the temperature of incipient melting is highly desirable.

Experimental Examples

In order to show that measurements are possible for biological products, we shall report here briefly some of our experimental data on horse serum and chick embryo extract.

For these two products, the resistivity increases quite regularly during the cooling period and, because of this fact, we have determined by experience that, when it has reached 10 megohms/cm. on an average, the substance could be considered thoroughly frozen. This condition corresponds for horse serum to −50° C. and for embryo extract to −56° C.

As for the "eutectic temperature," that is to say, the minimum temperature of incipient melting, it can be determined easily on the differential thermal analysis diagrams (FIGURES 15 and 16); this gives the following figures: horse serum, −34.5° C.; chick embryo extract, −17.5° C.

Of course, those products can be dried at higher temperatures, but in the case of horse serum we do not think that if total preservation of its qualities, especially its immunologic properties, is required, it is possible to dry this material at more than −34.5° C.

The Influence of Glycerol on the Freezing of Aqueous Systems: Vitreous Transformations and Devitrification

Another example of the use of thermal analysis to study the low-temperature evolution of freezing solutions can be given in the case of the glycerol-water system. I shall not develop that question, as it has been reported elsewhere (Rey, 1959*c*), but I shall point out some of the most important results and give complementary information.

It has been well known since the early work of Rostand (1946) and of Parkes *et al.* (1949) that the addition of a given amount of glycerol protects the living tissues against the deleterious effects of freezing. In our laboratory (Rey, 1958*b*) we have demonstrated that a chick embryo heart can be frozen at liquid nitrogen temperature without injury for several months after previous impregnation in a 30 per cent solution of glycerol in physiological saline. Under such conditions the heart presents, after thawing, a rhythmic activity with normal electric waves that can be recorded during several days when the organ is kept in organ culture at +38° C. (Rey, 1959*c*).

I have shown (Rey, 1959*d*) that the protective action of glycerol could be attributed to different factors. It decreases the crystallization velocity, dilutes the hypertonic solutions resulting from the separation of pure ice, facilitates supercooling, and permits the formation of amorphous structures at low temperatures.

To study this phenomenon more accurately, I have investigated the thermodynamic properties of simple glycerol-water solutions and have seen that between −80° C. and −196° C. very important solid reactions occurred in the frozen system.

Binary glycerol-water. Thermal analysis shows that the glycerol-water system presents a characteristic and reversible vitreous transformation at temperatures that vary with the concentration of glycerol (TABLE 3).

This transformation is followed, for all solutions except highly concentrated mixtures (over 90 per cent), by a slight exothermic phenomenon in the neighbor-

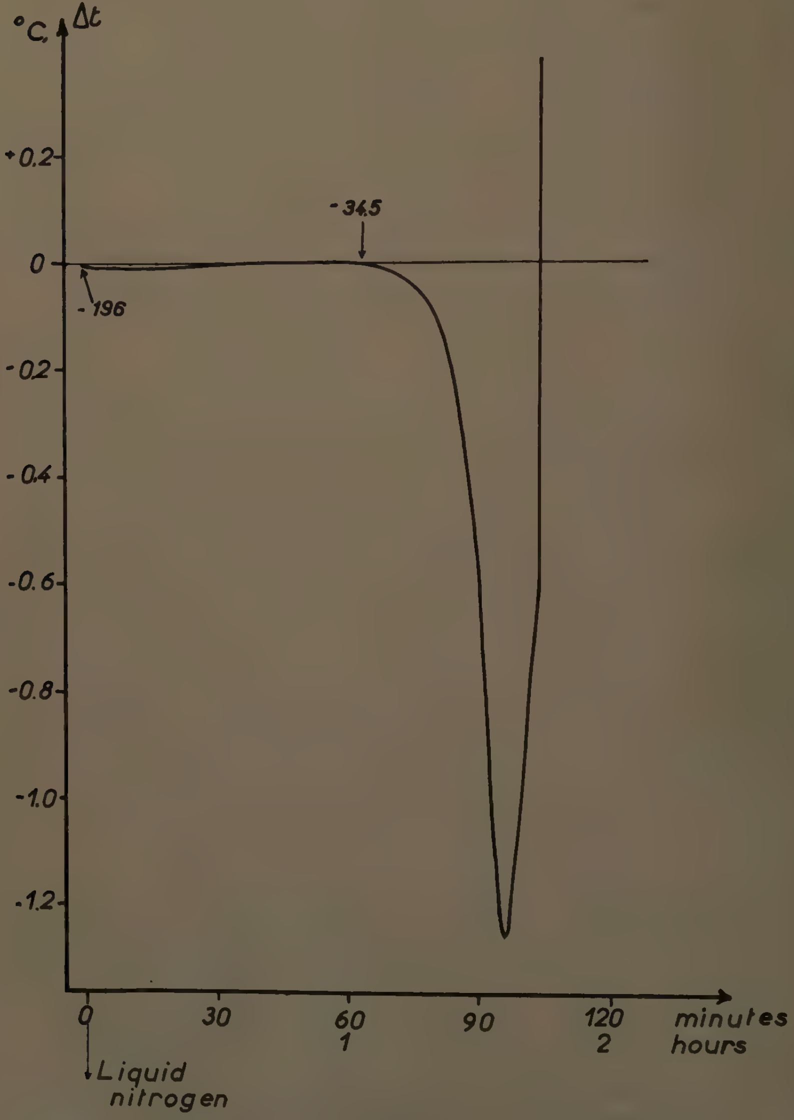

FIGURE 15. Differential thermal analysis diagram of normal horse serum.

hood of −80° C., but its exact temperature varies with the concentration of glycerol.

On an average, the solutions show a slow and continuous melting without any real eutectic phenomenon. However, for 66.7 per cent glycerol, there

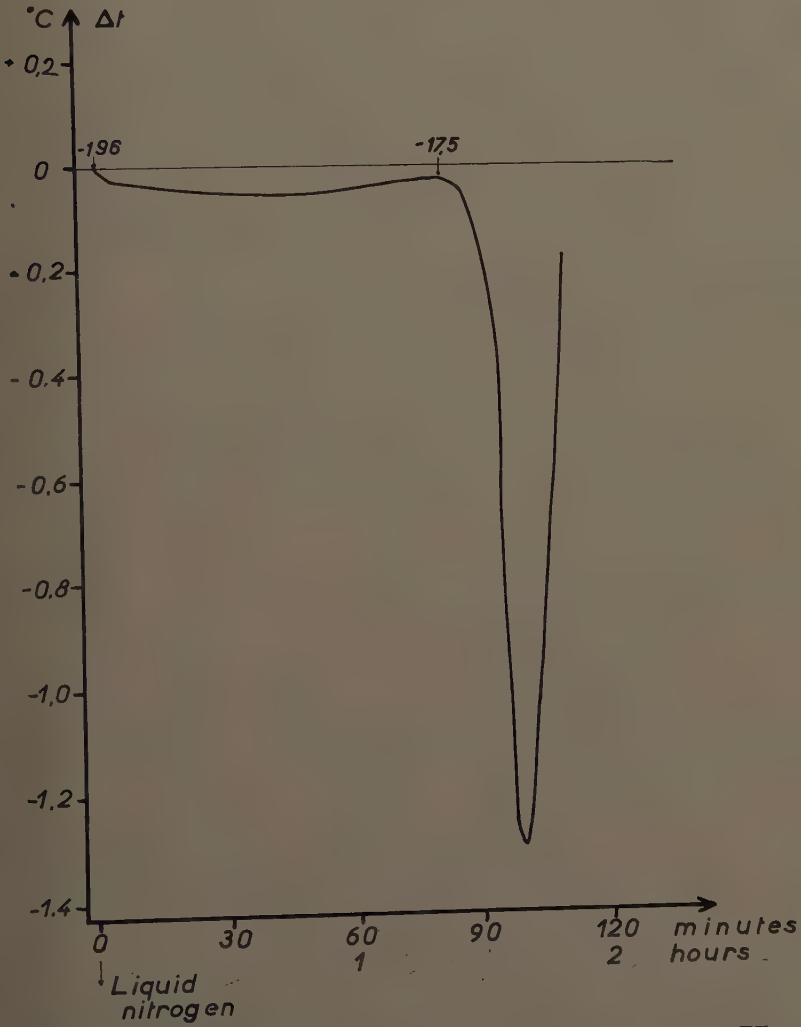

FIGURE 16. Differential thermal analysis diagram of chick embryo extract EE_{50}.

TABLE 3

VARIATION OF TRANSFORMATION TEMPERATURES WITH CONCENTRATION OF GLYCEROL

Percentage of glycerol	30	50	66.7	70	80	90	100
Temperature (°C.)	−108	−104	−112	−111	−104	−95	−86

occurs an important exothermic crystallization with a maximum at −61° C., which is followed by the characteristic thawing of the eutectic glycerol-water at −46.5° C. This may be seen also for 70 per cent and 80 per cent glycerol at −46.5° C., but it is markedly reduced.

As for the high percentages of glycerol (99 per cent and higher), they show the eutectic phenomenon only if the glycerol has been previously crystallized by thermal treatment.

Earle's balanced salt solution plus glycerol. Similar phenomena can be seen in a solution of glycerol in Earle's balanced salt solution (FIGURE 17), and for 50 per cent there is a marked crystallization peak at −105.5° C., following the vitreous transformation at −117° C.

It is likely that there is formation of a complex hydrate in which only a part of the glycerol participates. Actually, if the system is recooled from −80° C. to −196° C. and reanalyzed, the characteristic vitreous transformation of the binary glycerol-water may easily be seen.

We have found, in tissues that have been soaked in glycerol, that it is possible to find the same thermodynamic phenomena, although very much attenuated.

TABLE 4

VARIATION OF EXOTHERMIC PHENOMENON WITH CONCENTRATION OF GLYCEROL

Percentage of glycerol	30	50	66.7	70	80
Temperature (°C.) of exothermic phenomenon	−75	−94	−98	−88	−80

It thus appears that the low-temperature behavior of those tissues is dominated by the special properties of the glycerol-water system alone.

Metastable Equilibrium and Thermal Treatment

We have studied, thus far, freezing and thawing of solutions at low temperatures, chiefly in the light of crystallization and melting processes of ice alone or of more or less complicated eutectic systems.

However, the preceding comments on biological products and glycerol-water systems have shown us that many metastable equilibria can be reached during freezing. Many substances, such as glycerol-water solutions, do not crystallize entirely when they are frozen. The interstitial fluids concentrate progressively; their viscosity becomes higher and higher, and they soon assume a rigid state without the formation of crystalline structures. All this material hardens and becomes, at least partially, a glass. That glassy body is in a metastable state, but in certain circumstances it can persist indefinitely. When the material is rewarmed the reverse process occurs, and the glass progressively loses its high viscosity and slowly returns to the liquid state.

Of course, the products that yield such structures are very difficult to dry and generally, in the course of the lyophilization process, the continuous softening of the glass is the source of melting incidents with denaturation of the entire substance. Different sugars and alcohols, some vitamins, and many other compounds very often show such a low-temperature evolution. The formation of a glasslike structure is thus highly undesirable if the product that has been frozen is to be dried under vacuum.

By analogy to what we have seen in glycerol-water solutions, it was attempted, in those difficult cases, to apply a special thermal-treatment technique.

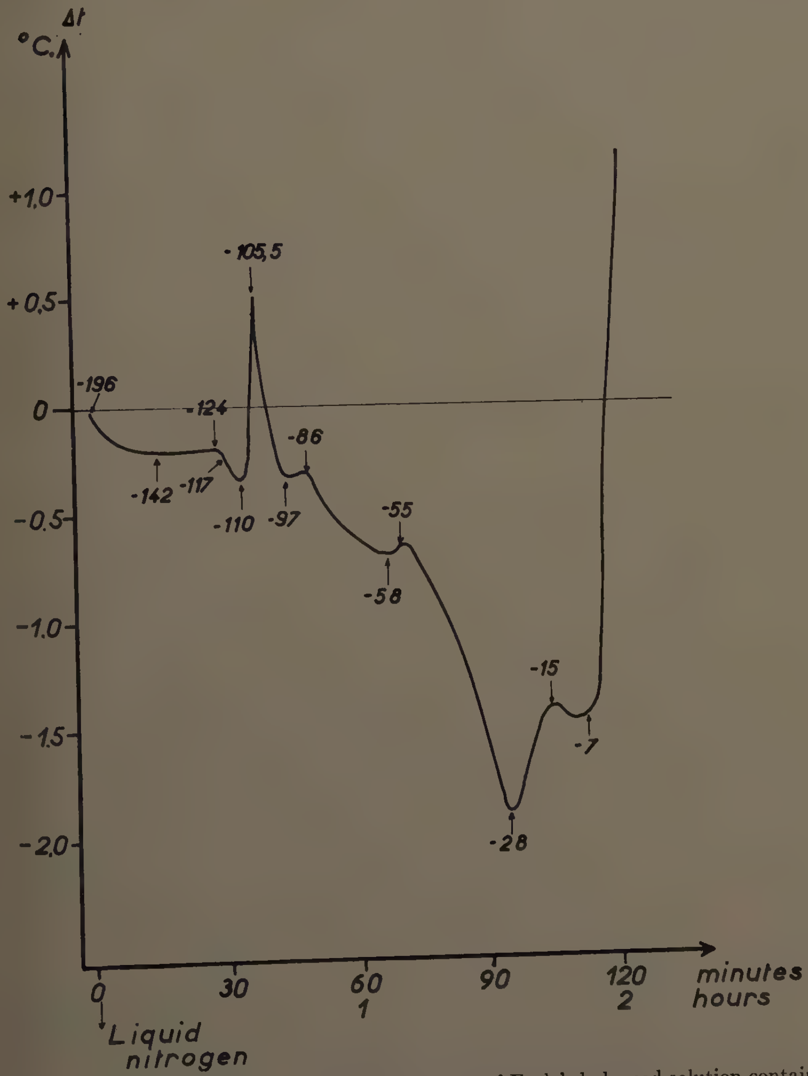

FIGURE 17. Differential thermal analysis diagram of Earle's balanced solution containing 50 per cent glycerol.

For that purpose it is necessary, first, to study very carefully the low-temperature thermodynamic properties of the system, both by resistivity and thermal analysis, and then, in the optimum case, when a devitrification temperature has been found, thermal treatment may be applied as follows:

In a first step a quick low-temperature freezing is performed in order to harden the solution and to allow the glassy structures to develop.

The material is then rewarmed very slowly and carefully up to the temperature of devitrification in order to induce crystal formation and to rupture the metastable equilibrium of the glass. Very often it is necessary to maintain the system for a long period at this temperature to be sure that the devitrification process will be completed (Rollet, 1935, 1936; Vuillard, 1957).

When this is done, the system is recooled and brought to the desired drying temperature, which has been determined largely by the position of the eutectic zone.

We have obtained very good results with this technique in our laboratory and, as far as industrial applications are concerned, the thermal treatment seems to be a very promising method for the drying of delicate products.

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ICE CRYSTAL FORMATION IN BIOLOGICAL MATERIALS DURING RAPID FREEZING

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In the rapid freezing of biological materials two closely related problems arise. The first is the dependence of the cooling rate of the sample on its size, shape, and thermal properties, on the velocity of immersion, and on the temperature and physical properties of the coolant. The second is the relation of the size and number of ice crystals to the thermal history of the sample. The primary complication in the analysis of these problems arises from the fact that the ice crystals, as they grow, release latent heat of crystallization. This heat release modifies the course of the cooling and, consequently, the temperature distribution in the sample. Conversely, the rate at which a given ice crystal can grow depends on its size and the temperature distribution of the surrounding aqueous medium.

Our present objective in the analysis of the situation is not to predict the thermal history of the sample from known values of the various physical parameters, but rather from measurements on the thermal history and various supporting data to gain information about the temperature dependence of ice crystal nucleation and growth rate. Two basic difficulties arise in doing this. The first is that, mathematically, we are forced to set up the problem as though we were going to compute the thermal history from basic constants and then go through a process of inversion. The second difficulty is that the amount of basic data at our disposal is rather limited. In a typical experiment we can determine the total number and size distribution of ice crystals at the end of cooling (insofar as they are of microscopic dimensions), and we can measure the temperature of one or several microthermocouples embedded in the sample. In order to convert this information into basic physical information it is always necessary to make rather sweeping simplifications. Clearly, then, the actual experimental situation must be so designed that these simplifications are reasonable approximations.

The first question is what relation the temperature of a thermal detector embedded in the sample has to the temperature of the surrounding sample. Clearly, any detector functions as an integrator of the thermal energy arriving at its own surface. The average temperature of the detector then will have some lag with respect to its surface temperature. The temperature lag for a small thermocouple,[2] 20 to 40 μ in diameter, is not serious; however, the averaging effect along its length is. Another complication is that, as soon as ice crystals begin to form throughout the sample, they act as numerous small heat sources, evolving heat as they grow in size. If these crystals grow with sufficient rapidity they can clearly cause local hot spots. In regard to these local hot spots one would like to know both how hot they can become and how rapidly heat released by one of them can be dispersed throughout the sample. If there is to be no significant temperature variation in the neighborhood of a growing ice crystal, the rate at which heat can be dispersed throughout the system must be much greater than the rate at which it is being released. In addition, if the heat released by freezing is to be reflected in the temperature of the detector, the rate of dispersion must be greater than the rate at which it can be released to the coolant. In other words, to make meaningful measurements on such a system the sample must be sufficiently small, and the heat transfer to the coolant must not be too efficient. In previous work, I have shown how the rate at which heat is released at the surface of the sample to the coolant satisfies the boundary condition

$$H(T_s - T_f) = -K \text{ grad. } T_s \tag{1}$$

where K is the heat conductivity of the sample and H depends on the coolant.[1,2] It is of the order of 0.2 cal./cm.^{2} sec. °C. for liquid propane and 0.02 cal./cm.^{2} sec. °C. for liquid nitrogen.

The basic mathematical solution for such a system is that for an arbitrarily placed instantaneous point source of unit strength. However, I shall use the slightly simplified solution for a concentric radial source of unit strength at $r = r'$ and $t = 0$. This is[3]

$$T(r) = \frac{1}{2\pi r r'} \sum_{n=1}^{\infty} \frac{(ah-1)^2 + a^2\alpha_n^2}{a^2\alpha_n^2 + ah(ah-1)} \sin r\alpha_n \sin r'\alpha_n e^{-\kappa\alpha_n^2 t} \tag{2}$$

where κ is the thermal diffusivity and the α_n are the roots of the transcendental equation

$$a\alpha \cot a\alpha + (ah - 1) = 0 \tag{3}$$

where $h = H/K$. For particular situations this solution must be integrated with respect to a temporal or spatial distribution of sources. In many of these problems a quantity of the order of $a^2/6\kappa$ occurs. This essentially represents the time lag between surface and center for surface cooling at a continuous rate (*see* APPENDIX I). The actual temperature difference is given by the rate of cooling times the time lag. For water we have

a	Time lag
1 cm.	100 sec.
0.1	1.0
0.01	0.01
0.001	0.0001

Frequently we have cooling rates of the order of 1000° C./sec.; hence the actual temperature variations in even very small samples can be large. However, if we restrict the cooling rate for a given size sample so that the temperature variation is small, we can write the integrated equation[2]

$$SH\Delta T/V + C_1\, dT/dt = L_f\, dw/dt - (C_2 - C_1)\,(dT/dt)w \tag{4}$$

in which we have neglected the volume expansion during freezing, and where S is the area of the surface bounding the specimen, V is its volume, ΔT the difference between sample temperature and coolant temperature, L_f is the latent heat of fusion, C_1 is the specific heat of water, C_2 is the specific heat of ice, and w is the fraction of the sample that has crystallized from water to ice. The above equation has the form

$$dw/dt + Pw = Q \tag{5}$$

where P and Q are known functions of time; that is, they can be computed from experimental data. This equation is a standard linear form and can be solved for w. If by a suitable experimental arrangement we are thus able to compute the fractional phase change w as a function of time, this can then be used, in a manner that I shall now discuss, to gain information about basic crystallization data: that is, the rates of nucleation and of crystal growth.

From a microscopic point of view the amount of water that has been transformed to ice at a given time is the sum of the volumes of the individual ice crystals. Each crystal is considered to start growing from a "nucleus" or "embryo" that has attained "critical size" as a result of statistical fluctuations. At time ω, between time t of measurement and zero time, let the rate of birth of critical size nuclei per unit mass of liquid be $N'(\omega)$ (*see* APPENDIX II). In general, N' will be strongly dependent on temperature distribution throughout the sample. If the temperature is uniform, $T(\omega)$, we have for the rate of birth of new nuclei per unit mass of liquid $N'[T(\omega)]$. If, however, fractional amount of liquid $w(\omega)$ has been transformed to solid, we must multiply by $[1 - w(\omega)]$ to obtain the rate of creation of new nuclei per unit mass of water and ice. If the average volume of an ice crystal (the reason for taking the average is to avoid the problem of overlap; *see* Jacobs and Tompkins[4] for further references) born at time ω, is $v(t,\omega)$ at time t, with mass $\rho v(t,\omega)$, where ρ is the density of ice; we have for the mass of water per unit mass converted to ice at time t

$$w(t) = \int_o^t N'[T(\omega)]\rho v(t,\omega)[1 - w(\omega)]\, d\omega \tag{6}$$

This is the basic integral equation for crystallization. If one knows $N'[T(\omega)]$ and $v(t,\omega)$ it is an equation in $w(t)$. In general, its solution offers great difficulty although, in certain special cases, it has been solved.[4] By like reasoning we get the relation for the number of nuclei at time t

$$N(t) = \int_o^t N'[T(\omega)][1 - w(\omega)]\, d\omega \tag{7}$$

From our present point of view, we regard $w(t)$ as an experimentally known function. Hence, EQUATIONS 6 and 7 are relations that must be satisfied by whatever values are theoretically predicted for $N'[T(\omega)]$ and $v(t,\omega)$ by microscopic kinetic theory (*see* APPENDIX II for a discussion of kinetic problems related to ice crystal nucleation and growth). Hence, good calorimetric data should be useful in testing various kinetic theories of crystal nucleation and growth.

APPENDIX I

In this appendix I discuss the time lag between surface and center of a sphere with constant cooling. This corresponds to cooling of a sphere with initial temperature T_o and surface temperature $T_o - kt$. We have the solution (Carslaw and Jaeger,[3] page 202)

$$T = T_o - k\left[t - \frac{a^2 - r^2}{6\kappa}\right] + \frac{2ka^3}{\kappa\pi^3 r}\sum_{n=1}^{\infty}\frac{(-1)^n}{n^3}\, e^{-\kappa n^2\pi^2 t/a^2}\sin\frac{n\pi r}{a} \tag{I,1}$$

From the above equation it can be seen that, when the transient is damped out, the time lag between center and surface is $a^2/6\kappa$. The average temperature of the sphere is given by

$$\mathrm{T} = T_o - k\left[t - \frac{a^2}{15\kappa}\right] - \frac{6ka^2}{\kappa}\sum_{n=1}^{\infty}\frac{1}{n^4}e^{-\kappa n^2\pi^2 t/a^2} \tag{I,2}$$

with a corresponding time lag of $a^2/15\kappa$. For many problems we have $k = dT/dt$, where dT/dt is the experimentally measured cooling rate; this can be seen by differentiating EQUATIONS I,1 or I,2.

For other geometries we have corresponding solutions. For a slab of half-thickness a, the lag between center and surface is $a^2/2\kappa$. For an infinite circular cylinder of radius a, the lag is $a^2/4\kappa$.

For other cooling problems, a quantity of this order of magnitude frequently appears. For example, if we integrate EQUATION 2 with respect to time for a continuous source of strength A, we find for $T(r)$ after the transients have disappeared,

$$T(r) = \frac{A}{2\pi rr'}\sum\frac{(ah-1)+\beta_n^2}{\beta_n^2 + ah(ah-1)}\,\frac{\sin r\alpha_n \sin r'\alpha_n}{\kappa\alpha_n^2} \tag{I,3}$$

For large n we have $\beta_n \cong n\pi$ [Consider the intersections of the graphs of $\cot\beta$ and $(ah-1)/\beta$]; hence $T(r)$ will be of the order of the expression

$$\frac{A}{2\pi rr'}\sum\frac{a^2}{\kappa n^2\pi^2}$$

which, since $\sum(1/n^2) = \pi^2/6$ (Knopp,[5] *Theory and Application of Infinite Series*, page 267), reduces to

$$\frac{A}{2\pi rr'}\,\frac{a^2}{6\kappa}$$

APPENDIX II

In this appendix we summarize certain considerations about ice crystal nucleation and growth. Recent detailed discussions of the general problem of solid-state transitions have appeared in the literature.[6-8]

Small embryos of solid arise in a supercooled liquid by statistical fluctuations in position and momentum of molecules of the melt. The free energy of formation of an embryo containing i molecules is given by

$$\Delta G = i(\mu_{II} - \mu_I) + a\sigma i^{2/3} \tag{II,1}$$

where μ_{II} is the chemical potential of the solid, in this case ice, μ_I is the chemical potential of the supercooled melt, σ is specific free surface energy between solid embryo and liquid, and a is a shape factor. If $\Delta G(i+1) > \Delta G(i)$, a nucleus is subcritical in size and will ordinarily decay. When $\Delta G(i+1) < \Delta G(i)$, the free energy of a nucleus is decreased by the addition of a molecule, and it can be expected to grow to macroscopic dimensions. We have:

$$\Delta G(i+1) - \Delta G(i) \lesseqgtr 0$$

according as

$$\mu_{II} - \mu_I + a\sigma(i+1)^{2/3} - a\sigma i^{2/3} \lesseqgtr 0$$

approximately as

$$\mu_{II} - \mu_I + \tfrac{2}{3}a\sigma i^{-1/3} \lesseqgtr 0$$

Hence, the number of molecules in the critical nucleus dividing the two regions is approximately

$$i^* = -\left(\frac{2a\sigma}{3(\mu_{II} - \mu_I)}\right)^3 \tag{II,2}$$

and the corresponding free energy is

$$\Delta G^* = \frac{4a^3\sigma^3}{27(\mu_{II} - \mu_I)^2} \tag{II,3}$$

At the equilibrium temperature between bulk liquid and bulk solid, T_o, we have $\mu_{II} = \mu_I$; also from the relation $S = -\partial G/\partial T$ we find

$$\mu_I - \mu_{II} = \Delta S(T_o - T) = \Delta H(T_o - T)/T \tag{II,4}$$

where ΔS is the entropy of freezing per molecule and ΔH is the heat of freezing per molecule. Substituting EQUATION II,4 in EQUATION II,2 and EQUATION II,3, we obtain

$$\Delta G^* = \frac{4a^3\sigma^3}{27(\Delta S)^2(T_o - T)^2} \tag{II,5}$$

and

$$i^* = \left(\frac{2a\sigma}{3\Delta S(T_o - T)}\right)^3 \tag{II,6}$$

For a spherical nucleus we have the equations

$$r^* = \frac{2\sigma T_o}{L_f\rho(T_o - T)} \tag{II,7}$$

and

$$\Delta G^* = \frac{16\pi\sigma^3 T_o^2}{3\rho^2 L_f^2(T_o - \mathrm{T})^2} \tag{II,8}$$

where σ is interfacial tension per cm.^{2}, L_f heat of fusion per gram of ice, ρ ice density, and T absolute temperature. If, anticipating the following discussion, we substitute values for pure water and ice: $\rho = 0.917$ gm./cm.^{3}, $\sigma = 25$ ergs/cm.^{2}, $L_f = 3.35 \times 10^9$ ergs/gm., $T_o = 273°$ K., we find

$$r^* \cong \frac{500}{(T_o - T)} 10^{-8} \text{ cm.} = \frac{500}{T_o - T} \text{Å}. \tag{II,9}$$

It will be observed that the foregoing is all classic equilibrium theory and is valid in so far as the shape factor a and σ are known.

Turnbull,[8] utilizing Eyring's theory of absolute reaction rates,[9] has shown that the net forward rate at which an equilibrium distribution of critical nuclei becomes larger than critical and hence actively growing is

$$\frac{dN}{dt} \cong n_1 \frac{kT}{h} \exp\left[-(\Delta g^* + \Delta G^*)/kT\right] \tag{II,10}$$

where n_1 is the number of molecules per unit volume of liquid, k is Boltzmann's constant, h is Planck's constant, and Δg^* is the free energy of activation for diffusion of molecules across the liquid crystal interface. It is very difficult to compute precise values of dN/dt from the above equation, because the pre-exponential factor is only approximate, Δg^* is usually not known precisely, and neither is ΔG^* because of the surface tension term σ, which enters into its computation. In addition, there is a nucleation time lag[7] before the equilibrium state is established, which may be very important, particularly in colloidal systems. However, EQUATION II,10 has been very useful in the discussion of nucleation problems. The expression is dominated by the factor exp $[-\Delta G^*/kT]$ and, from measurements of dN/dt as a function of temperature, ΔG^* and therefore σ can be determined.[10,11]

The application of the above theory to nucleation in biological systems requires modification. In the first place, it is not clear whether ice crystal nuclei in such systems are homogeneous or whether they are heterogeneous; that is, if they form on the surface of colloidal particles. Either case will give rise to an expression similar in form to EQUATION II,10, but the constants appearing in Δg^* and ΔG^* will be different than those for pure water. In the absence of experimental data one can only speculate on the magnitude of the difference. A relatively small difference in the value of $\Delta g^* + \Delta G^*$ can cause a large difference in the absolute value of dN/dt, but not very much in the value of r^*.

In particular, the value of the activation energy for diffusion Δg^* will be greatly different in colloidal solutions. This is ordinarily taken as the activation energy for diffusion of the most slowly moving component of the system.

The theory of the crystal growth of nuclei supercritical in size is even less developed than the theory of nucleation. As an ice crystal grows in size, several concurrent phenomena must occur. Heat of crystallization must diffuse away from the crystal. Water molecules must cross the interface between crystal and melt to become part of the crystal. Moreover, colloidal particles in a colloidal system are not incorporated into the ice crystal, but are swept ahead of its growing faces. These particles must diffuse away from the crystal; otherwise, they will reach a concentration such that precipitation will occur. The crystal will then be surrounded by a dense colloidal matrix through which water molecules must diffuse if further growth is to occur. Some of these phenomena can be formulated mathematically, but they fall into the general class of unsteady-state diffusion or heat-conduction problems with moving boundary. Solutions for this type of problem have been obtained only for very special cases.[12] It is apparent, however, that the rate of growth of ice crystals in colloidal systems should be substantially reduced relative to the growth in water.

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ICE PROPAGATION IN GLYCEROL SOLUTIONS AT TEMPERATURES BELOW −40° C.*

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Introduction

Limited information is available regarding the effects of solutes on ice propagation in biological materials at temperatures below −40° C. Previous dilatometric studies showed that, in the absence of nucleating agents, ice formation was not detectable in 50 ml. of 15 molal glycerol cooled at 0.25° C./min. to a final temperature of −100° C. (Lusena and Cook, 1954). It was suggested that, as the concentration increases during freezing, the heat of crystallization may become insufficient to prevent a rapid temperature drop to a region where nucleation and crystal growth are suppressed. Almost static small ice crystals can thus be interspersed within a supercooled solution.

This paper describes a dilatometric method for studying ice formation at low temperatures and provides more information on ice propagation at temperatures below −40° C. in glycerol solutions. Glycerol was chosen for this investigation because it is commonly used to prevent freezing injury in biological materials (Smith, 1954), and because it does not crystallize from aqueous solutions without nuclei (Miner and Dalton, 1953).

Methods

Dilatometric technique. The principle of the method was based on two observations: first, ice formation is essentially stopped at temperatures below −120° C. (Burton and Oliver, 1935; Meryman and Kafig, 1955; Pryde and Jones, 1952), and second, 30-ml. volumes of glycerol solutions more concentrated than 50 per cent could be rapidly cooled to −140° C. without visible ice formation. Therefore, the difference in volume between solutions quickly cooled to −140° C. and those cooled to −140° C. after exposure for various periods at higher temperatures could be used as a measure of the ice formed at higher temperatures.

The glass dilatometer used consisted essentially of a 50-ml. bulb, a reservoir, and a calibrated capillary (FIGURE 1). About 35 gm. of glycerol solution was weighed into the bulb, which was then sealed to the S-shaped reservoir and filled with 99 per cent isopentane. The air was removed by repeated freezing to −79° C. and slow thawing until no air was released in at least 5 successive freezings. The reservoir was sealed to the calibrated capillary, and the complete system was filled with isopentane. The bottom of the reservoir, the capillaries, and part of the wide extension were filled with mercury so that, on cooling quickly to −140° C., the mercury was at about the 10-cm. level in the calibrated capillary.

The mercury level in the capillary was read with the bulb, and a fixed portion of the stem between bulb and reservoir immersed in a well-stirred isopentane bath maintained at −140° ± 0.2° C. (FIGURE 1). The whole dilatometer was then exposed to the desired higher temperature for a period of time, returned to the same position in the −140° C. bath, and the mercury level redetermined. Readings became constant within 1 hour. An experimentally determined correction of about 0.01 ml. per degree centigrade compensated for temperature variations (−8° C. to 4° C.) in the reservoir.

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The change in volume of the sample caused by the difference between the volume of the unfrozen portion of glycerol solution plus the volume of ice at −140° C. and the volume of original solution at −140° C. can be expressed as

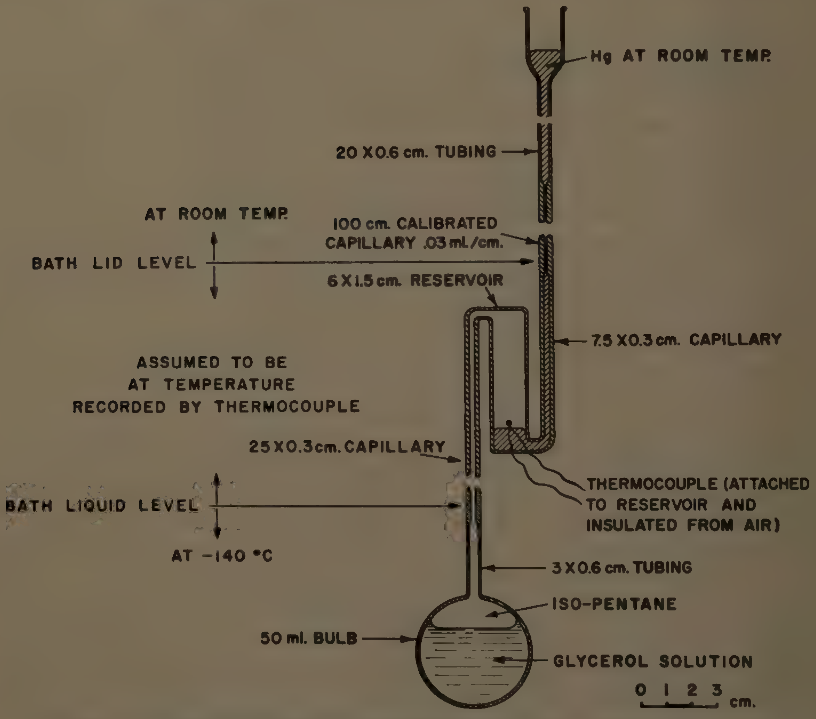

FIGURE 1. Schematic diagram of the dilatometer used in these experiments.

$$\Delta V = (W_s - W_i)\,S_u + W_i S_i - W_s S_s$$

(symbols defined in TABLE 1). However, the difference in capillary reading is the sum of volume increase of: (1) the sample, (2) the isopentane moved from the bulb at −140° C. to the reservoir at 0° C., and (3) the mercury moved from the reservoir to the capillary at room temperature. The last 2 changes in volumes are proportional to the first, and the correction factor (K) of 0.829 was determined for a reservoir temperature of 0° C. and a room temperature of 23° C. K varied by about 0.001 for each degree of temperature change in the reservoir, and by about 0.0003 for each degree change in room temperature.

Since

$$\Delta V = KC\Delta L$$

and

$$W_i = W_s - \frac{100\, W_g}{G}$$

then

$$\Delta L = \frac{W_s(S_i - S_s) - \dfrac{100\, W_g}{G}(S_i - S_u)}{CK}$$

The specific volume of glycerol solutions at −140° C. was determined pycnometrically, using isopentane with a density of 0.7685 at −140° C. (Timmermans, 1912) for calibration. This value was confirmed (D. W. Davidson, private communication) for the isopentane used in this study. The pycnometers consisted of two 50-ml. bulbs connected by a capillary and with a filling stem on the upper bulb. A known amount of liquid being tested was placed in the lower bulb with a hypodermic needle to avoid wetting the upper bulb. The pycnometer was cooled to −140° C. and isopentane added to the level of a mark in the capillary. The lower bulb was then immersed in liquid nitrogen, the filling stem sealed with a torch, and the sealed pycnometer equilibrated to room temperature and weighed. Thirty determinations on various solutions containing between 52 and 74 per cent glycerol yielded a smooth curve (FIGURE 2). Uncertainty in drawing the curve increased with increasing concentration;

TABLE 1

DEFINITION OF SYMBOLS, UNITS, VALUES, AND MAXIMUM ERRORS

Symbol	Definition	Unit	Value or range of values	Maximum error*
ΔV	Change in volume	ml.	—	—
ΔL	Difference in capillary reading	cm.	0–50	1.0
C	Capillary calibration	ml./cm.	0.0281–0.0317	0.00005
K	Correction factor	ml./ml.	0.829	0.008
W_s	Weight of sample	gm.	32.9–34.7	0.001
W_g	Weight of glycerol	gm.	18.2–21.9	0.03
W_i	Weight of ice	gm.	—	—
G	Glycerol concentration in unfrozen portion	%(w/w)	63–79	0.4
S_s	Specific volume of sample at −140° C.	ml./gm.	0.826–0.808	0.0002–0.0007†
S_u	Specific volume of unfrozen portion at −140° C.	ml./gm.	0.808–0.784	0.0007–0.0015†
S_i	Specific volume of ice at −140° C.	ml./gm.	1.0701	0.002‡

* Maximum random experimental differences that do not include the systemic possible error in the constants chosen.

† The significance of these ranges of differences is discussed in the text.

‡ This is the maximum difference from the mean of 3 acceptable reported values (Jacob and Erk, 1928; Dorsey, 1940; Tessier *et al.*, 1956).

hence the range of maximum error reported in TABLE 1. No ice was detected during these determinations.

From the above equation, S_u being a known function of G, ΔL could be calculated for assumed values of G for each dilatometer and plotted against G. Within the range of glycerol concentrations of interest, ΔL was a linear function of G with a slope of about 0.4 per cent per centimeter. Hence, values of G could be determined easily from measured values of ΔL.

The maximum possible error, obtained by summation of the individual deviations of the factors in TABLE 1, in the concentration of the unfrozen portion at −79° C. is about 4 per cent. Duplicate determinations always agreed within 0.4 per cent glycerol concentration.

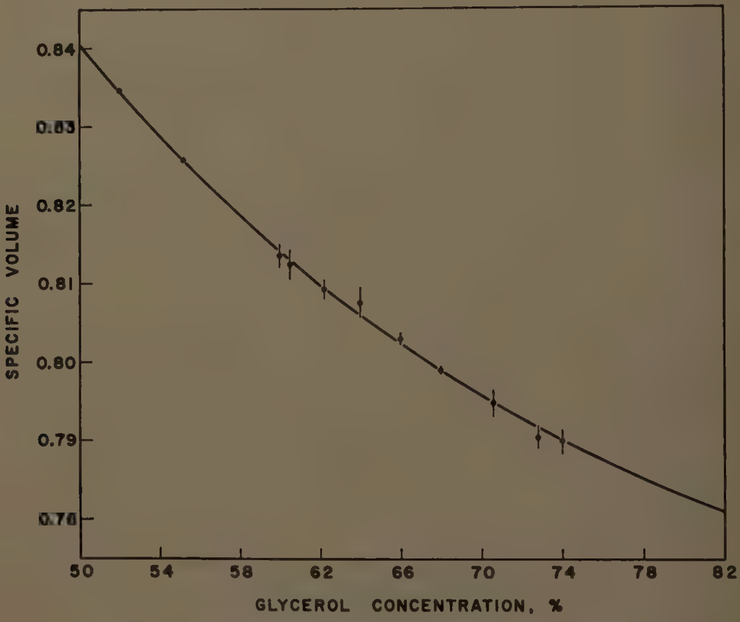

FIGURE 2. Specific volumes of glycerol solutions at −140° C.

Experimental Technique

Solutions containing 55, 59, and 63 per cent glycerol (the latter with and without the addition of hexagonal silver iodide crystals) were cooled quickly to −140° C., and the mercury level was recorded. No visible ice was formed in solutions of 59 and 63 per cent glycerol, but a fine misty layer of ice was observed at the interface between 55 per cent glycerol solution and isopentane. However, no change in volume was observed over a 3-day period at −140° C., and the readings were reproducible. The increase in volume that resulted when these dilatometers were placed for periods varying from 0.25 to 336 hours in a well-stirred bath at −79° C., −55° C., or −40° C. (±0.2° C.) and then returned to −140° C. was determined.

Results and Discussion

The equilibrium concentration in the unfrozen portion of 4 glycerol solutions stored at −79° C. for various lengths of time (TABLE 2) was about 79.1 per cent. This concentration was reached in less than 15 min. for the 55 per cent glycerol solution, in more than 2 hours for the 59 per cent solution, and in more than 65 hours for the 63 per cent solution even in the presence of hexagonal silver iodide crystals. Since the rate of ice formation is primarily controlled by the number of nuclei and the rate of crystal growth, and since the rate of crystal growth must be the same once each solution has reached the same concentration, the great difference in rate of ice formation must be caused by differences in number of nuclei. It is probable that much of the nucleation occurred during the original quick cooling to −140° C.

TABLE 2

EFFECT OF STORAGE TIME AT −79° C. ON ICE FORMATION

Samples at −140° C. When Placed in −79° C. Bath: Averages of Duplicate Determinations

Time in −79° C. bath (hours)	Glycerol concentration in unfrozen portion (per cent)			
	55% solution	59% solution	63% solution	63% solution*
0.25	79.1	59.2	63.2	63.0
0.5	79.1	59.8	63.1	63.0
1.0	79.3	63.4	63.3	63.5
2.0	79.4	77.6	64.5	64.5
3.5	79.1	79.2	70.4	71.4
17.0	78.9	79.1	78.0	78.5
65.0	79.1	79.2	78.1	78.7
336†	—	—	79.1	79.2

* Containing silver iodide.
† Single determination.

TABLE 3

EFFECT OF STORAGE TIME AT −55° C. ON ICE FORMATION

Samples at −140° C. When Placed in −55° C. Bath

Time in −55° C. bath (hours)	Glycerol concentration in unfrozen portion (per cent)			
	55% solution	59% solution	63% solution	63% solution*
0.25†	77.0	75.9	65.1	65.3
0.5†	76.1	75.5	73.0	73.4
1.0	74.5	74.8	72.5	72.8
2.0	74.4	74.6	72.7	73.0
3.5	73.6	73.8	72.1	72.5
7.0	72.8	73.4	71.8	71.8
14.0	72.3	72.8	71.7	72.0

* Containing silver iodide.
† Averages of duplicate determinations.

The equilibrium concentration in the unfrozen portion at −55° C. was about 72.0 per cent (TABLE 3). During warming from −140° C. to −55° C. the samples reached −65° C. in about 15 min. and approached −55° C. in an additional 15 min. The concentration developed at these lower temperatures required considerable time to change to the equilibrium concentration. Significant amounts of ice could not be formed during the converse process, that is, cooling from −55° C. to −140° C., because the center of the dilatometer reached −90° C. in about 1 min.

TABLE 4
EFFECT OF STORAGE TIME AT −40° C. ON ICE FORMATION
Samples at −140° C. When Placed in −40° C. Bath

Time in −40° C. bath (hours)	Glycerol concentration in unfrozen portion (per cent)			
	55% solution	59% solution	63% solution	63% solution*
0.25	73.9	73.6	64.9	64.8
0.5	72.8	72.0	65.1	65.0
1.0	71.5	70.5	63.4	63.4
2.0	70.4	69.2	63.2	63.0
3.5	68.3	68.1	63.0	63.3
19.0	66.8	66.0	63.0	63.0
66.0	66.3	64.6	62.9	63.0
336.0	65.7	63.5	63.2	63.3

* Containing silver iodide.

TABLE 5
EFFECT OF STORAGE TIME AT −40° C. ON ICE FORMATION
Samples at Room Temperature When Placed in −40° C. Bath

Time in −40° C. bath (hours)	Glycerol concentration in unfrozen portion (per cent)			
	55% solution	59% solution	63% solution	63% solution*
1†	67.7	59.6	63.1	63.0
2†	69.3	61.7	63.0	63.1
3.5‡	68.5	62.6	63.1	63.2
17	66.0	63.7	63.0	63.1
66	65.0	63.3	63.2	63.2
240	63.6	63.3	63.1	63.0

* Containing silver iodide.
† Averages of duplicate determinations.
‡ Averages of triplicate determinations.

The equilibrium concentration in the unfrozen portion at −40° C. was about 63.3 per cent (TABLES 4 and 5). Higher concentrations in the samples stored at −40° C. after equilibration at −140° C. (TABLE 4) developed in part on warming, but the results at −55° C. (TABLE 3) indicate that equilibrium would be reached in less than 10 hours at −40° C. Higher concentrations in these samples (TABLE 4) and in the samples equilibrated at room temperature (TABLE 5) also developed on recooling to −140° C., the time of exposure in the region of ice growth being much longer on cooling from −40° C. than from −55° C. The amount of ice formed during temperature transition also depends on the ratio of ice surface to amount of unfrozen solution and therefore on the size of ice crystals. On storage, ice crystals tend to increase in size and, when they are sufficiently large, the extra ice formed during cooling becomes insignificant. This critical size was reached considerably faster by the room-temperature samples (TABLE 5) than by the −140° C. samples (TABLE 4), because the initial ice crystals formed at a higher temperature and were therefore larger.

Since all results were consistent, it can be concluded that the various constants and assumptions used in the calculations were sound and that air was adequately removed. Although the ice haze that formed during the preliminary cooling to −140° C. was dependent on the original glycerol concentration, all solutions gave the same equilibrium concentration. Therefore, the amount of haze was not significant by comparison with the total ice formed at various temperatures.

From the equilibrium concentrations the molal depressions of the freezing point can be calculated (TABLE 6). The value at −40° C. is in excellent agreement with the published phase rule diagram, and the values below the eutectic point fit its extrapolation (Lane, 1925).

TABLE 6

MOLAL DEPRESSIONS OF FREEZING POINT AS CALCULATED FROM EQUILIBRIUM CONCENTRATIONS

Freezing temperature (°C.)	Glycerol concentration in unfrozen portion		Molal depression of freezing point (°C./mole)
	Per cent	Molal	
−79	79.1	41.1	1.93
−55	72.0	27.9	1.97
−40	63.3	18.7	2.13

Since the addition of silver iodide did not alter the results, homogeneous nucleation must be more important than heterogeneous nucleation at the concentrations and temperatures used.

Under the conditions of these experiments, 2 days may be required to complete freezing (TABLE 2) and one half a day to thaw the ice formed on warming (TABLE 3), but the crystal growth by redistribution, as judged by ice formation on cooling to −140° C., was still occurring after 2 weeks at −40° C. and may have continued for much longer periods. These results show conclusively that data on the freezing of biological materials cannot be interpreted if only bath temperatures are recorded. A supercooled phase may persist for long periods in the presence of ice and, conversely, excess ice formed during rapid freezing at low temperatures (for subsequent storage at higher temperatures) may take a long time to melt. Furthermore, the ice redistribution that occurs during storage affects the ice formed during subsequent temperature changes. These findings are probably not specific to glycerol solutions, as it has been shown that lactose (Tessier *et al.*, 1956) and sucrose do not crystallize easily below the eutectic point. Sucrose did not crystallize from solutions after 3 months' storage at −20° C. (Lusena, unpublished results).

Summary

A dilatometric method has been developed that measures the difference in volume between samples cooled rapidly to −140° C. without freezing and samples frozen at specific temperatures and then cooled to −140° C. From this measurement the amount of ice formed at temperatures below −40° C. in solutions of more than 50 per cent glycerol concentration is calculated. The required specific volumes of glycerol solutions at −140° C. are reported.

Rates of formation, dissolution, and redistribution of ice at temperatures below −40° C. were found to be very slow in these glycerol solutions. As much as 2 weeks was required to attain equilibrium after a change in temperature. Molal depressions of the freezing point for glycerol at −40° C., −55° C., and −79° C. were 2.13, 1.97, and 1.93, respectively. The value at −40° C. is in excellent agreement with a published phase rule diagram, and the values below the eutectic point fit its extrapolation. The persistence of nonequilibrium conditions and the long time required for ice redistribution show that results on the freezing of biological materials cannot be interpreted adequately if only the bath temperatures are recorded.

Acknowledgments

Appreciation is expressed to Fay Inglis and Dorothy Stotesbury for their technical assistance.

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ON VARIOUS PHASE TRANSITIONS OCCURRING IN AQUEOUS SOLUTIONS AT LOW TEMPERATURES

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One of my chief objectives in this paper is to point to a unifying principle, namely, the predominant effect of cooling velocity, in the multiple transformations that take place in aqueous solutions at low temperatures (between 0° and −195° C.). The transformations examined here have been a subject of investigation in this laboratory for several years, and the material on which the paper is based is taken mostly from our work. The occurrence of phase transitions in aqueous solutions is considered here from the viewpoint of the biologist who seeks, in the freezing of simple physical systems, some basic information on the mechanisms of injury by freezing and of prevention of that injury.

The expression phase transitions includes primarily the passage from the liquid, amorphous state into the crystalline state, but also miscellaneous other transitions, such as solidification in the amorphous state, transformations within the amorphous state (such as the so-called glass transition), various forms of incomplete crystallization (some of which have been designated in the literature as vitrification), various types of "recrystallization," and the passage from one crystalline system to another.

Investigations on phase transitions in aqueous solutions presuppose a knowledge of the transitions undergone by pure water. I shall therefore examine, in a first part of this paper, the information that we have on water, taking it primarily from the literature. In the second part I shall present the data on solutions, mostly as supplied by our own observations. Each of these two parts will consist of two sections: one (A) treating typical crystallization, and the second (B) treating miscellaneous other transitions.

Since practically all solutions encountered in biological processes are dilute, their solidification takes place in two steps: a first, called hereafter primary crystallization, in which the solvent (water) freezes out, while the solutes become more concentrated, and a second, designated as secondary crystallization, in which the components of the concentrated solution solidify (precipitation of the solutes, or of their hydrates, or simultaneous crystallization of the solute-solvent mixtures, in particular of the eutectic mixture). There will thus be two stages to examine under crystallization of solutions: (a) the primary, and (b) the secondary crystallization.

The general division of the paper will then be as follows:

- (1) Water
  - (A) Typical crystallization
  - (B) Miscellaneous transitions
- (2) Solutions
  - (A) Typical crystallization
    - (a) Primary crystallization
    - (b) Secondary crystallization
  - (B) Miscellaneous transitions

The principal features studied in our long-range research program were: (1) the number of crystallization centers formed in a given volume and, when none was formed, the degree of supercooling; (2) the type of crystalline formations encountered and their mode of development; (3) the rate of growth of the crystalline phase; (4) the modifications undergone by crystalline formations under various conditions, especially under the effect of inhibiting factors, and the changes in the nonfrozen phase, especially in solute concentration, resulting from freezing; and (5) the total amount of ice or crystalline solid formed. Of these five groups of characteristics, only the second and some of the fourth, that is, the type of crystalline formations and the modifications and inhibitions of crystallization, are examined in this paper.

The principal factors studied in the research program and considered here are: (1) the cooling velocity (which depends primarily on the temperature of the cooling bath and on the dimensions and geometry of the specimen and of its supports), (2) the nature of the solute, and (3) the concentration of the solute.

(1) Phase Transitions in Water

(A) TYPICAL CRYSTALLIZATION

The molecule of water as the building block of the ice crystal. Modern physicists and physical chemists have been quite successful in accounting for most of the properties of the molecules by an analysis of their electronic distribution. The application of the wave theory of the electron, of the theory of quanta, of the principle of uncertainty, of the theory of the sharing of electrons in chemical binding, and of that of the pairing of electrons has led to a partial explanation of the geometrical arrangement of the forces that cause atomic or molecular aggregation. The conclusion of such studies, as well as of previous ones, such as that of the polar properties of molecules, has been that the molecule of water, the morphology of which can be represented with a fair degree of accuracy as in FIGURE 1*a*, has 4 centers of attraction or repulsion: A, B, C, and D. It behaves as if it were carrying 2 positive charges on the sides of the hydrogen atoms (marked +), at A and B, and 2 negative charges on the side of the atom of oxygen (marked −), at C and D; thus, each of the 2 points A and B of any molecule may attract one of the 2 points C and D of another molecule. The consequence is that the molecules attach to one another and, in doing so, build up the crystalline structure.

Construction of the ice crystal. A crystal of ice, which has the shape of a hexagonal prism, may be considered as consisting of layers of molecules, the "basal planes," superimposed on one another, as shown in FIGURE 1*e*, and the basal planes themselves may be considered as composed of elementary hexagons arranged as in FIGURE 1*d*. The crystal may also be considered as consisting of zigzagging chains of molecules (of the type shown in FIGURE 1*b*), or of tetrahedra (such as that drawn in FIGURE 1*c*), arranged in a particular way in the 3 spatial directions. It is possible that such groups as the chains, the hexagons, or the polyhedra are preformed and that in the actual construction of the crystal they become attached, as whole blocks, to the growing crystalline edifice.

Growth of ice crystals and formation of crystalline skeletons. In studying cases of slow growth of ice crystals in solutions we could observe at leisure what appears to be the mechanism of growth in crystals of pure ice and in crystals in general. I mention here only a few observations concerning the mode of aggregation of the molecules of water to the crystalline frame.

When a solid hexagonal plate has been formed around a center of crystallization (FIGURE 2*a* and FIGURE 3*a*), the 6 corners grow faster than the faces, thus leading to the formation of stars (FIGURE 2*b* and FIGURE 3*b*), and next, of hexagonal rosettes (FIGURE 2*c* and *d*, and FIGURE 3*c*). Then branches grow at 60° angles from the 6 axial stems, secondary branches at 60° angles from the primary ones, and so on (FIGURE 2*e* and FIGURE 3*d*), and a "skeleton" is thus built that still has the hexagonal form (FIGURE 2*f*).

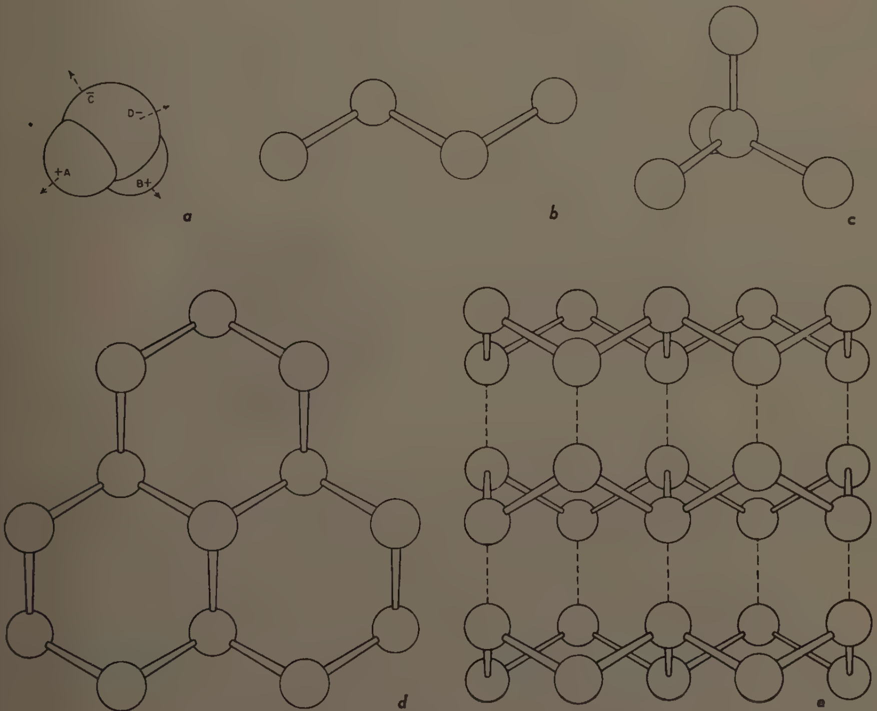

FIGURE 1. Diagrams illustrating the structural components of ice crystals. (*a*) Molecule of water with its centers of attraction A, B, C, and D. (*b*, *c*, and *d*) Arrangement of molecules of water into a chain, a tetrahedron, and hexagonal figures, respectively. (*d*) Arrangement of hexagonal figures into a "basal plane." (*e*) Superimposition of basal planes into a prismatic structure. In *a* the spheres represent the atoms of oxygen and hydrogen; in *b* to *e* they represent molecules of water. Reproduced from Luyet and Rapatz, 1958, by permission of *Biodynamica*.

Since all the directions of growth of the stems and branches in a skeleton are along the 3 axes or parallel to them (FIGURE 2*g* and FIGURE 3*d*), it follows that the molecules of water attach themselves to the crystal only along those preferred directions.

Dimensions and rate of growth. Since in ice the distance between the centers of the molecules of water is about 3 Å, the materials needed for the construction of a piece of ice measuring a micron cube would be 27 billion molecules

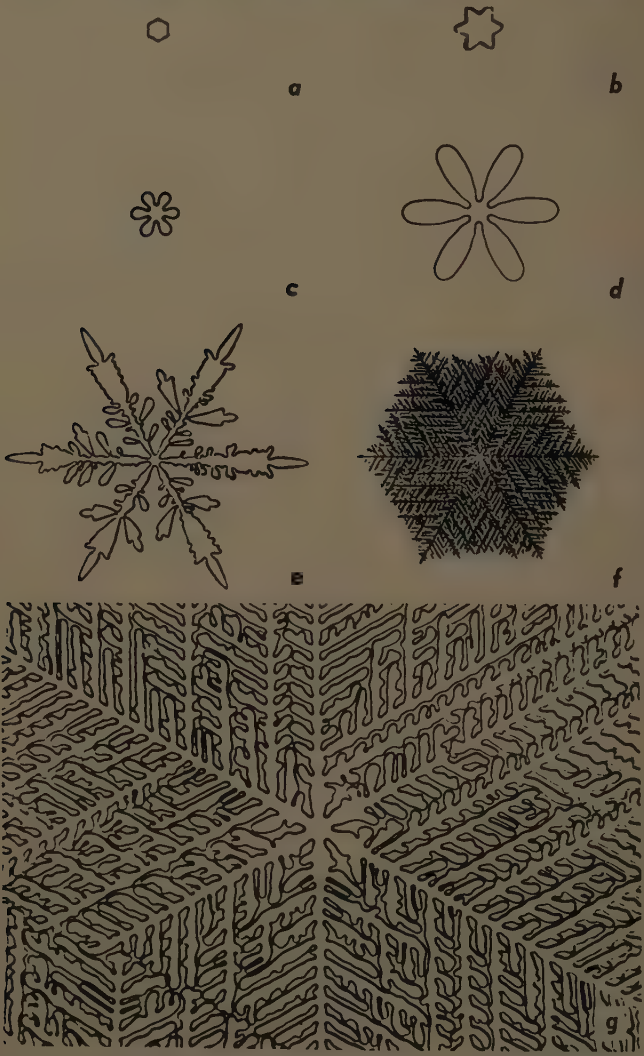

FIGURE 2. Hexagonal ice formations obtained in a 35 per cent albumin solution at high subzero temperatures. (*a*) Hexagonal plate; (*b*) star; (*c* and *d*) hexagonal rosettes; (*e*) development of branches; (*f*) skeleton; (*g*) details of skeleton. ×95. Reproduced from photographs by Luyet and Rapatz, 1958, by permission of *Biodynamica.*

(3000 molecules in a row to cover a length of 1 μ, 3000 such rows to cover an area of 1 μ square, and 3000 such layers to build up a cube of 1 μ on the side; $3000^3 = 27$ billions).

The activity of the molecules in the construction of crystalline edifices is tremendous for an observer accustomed to human activity and using the human time scale. A task involving the arrangement of billions of construction blocks may be completed in a small fraction of a second.

Structural disturbances and freezing velocity. The structure of the ice crystal described thus far is that which would be obtained under ideal conditions. The closest approach to that ideal is realized when freezing proceeds slowly and the molecules are given time to take their proper position in the structure. Increasing the freezing velocity results in an infinite variety of structural disturbances. It is precisely these disturbances and the factors responsible for them that are the subject of the major part of this paper.

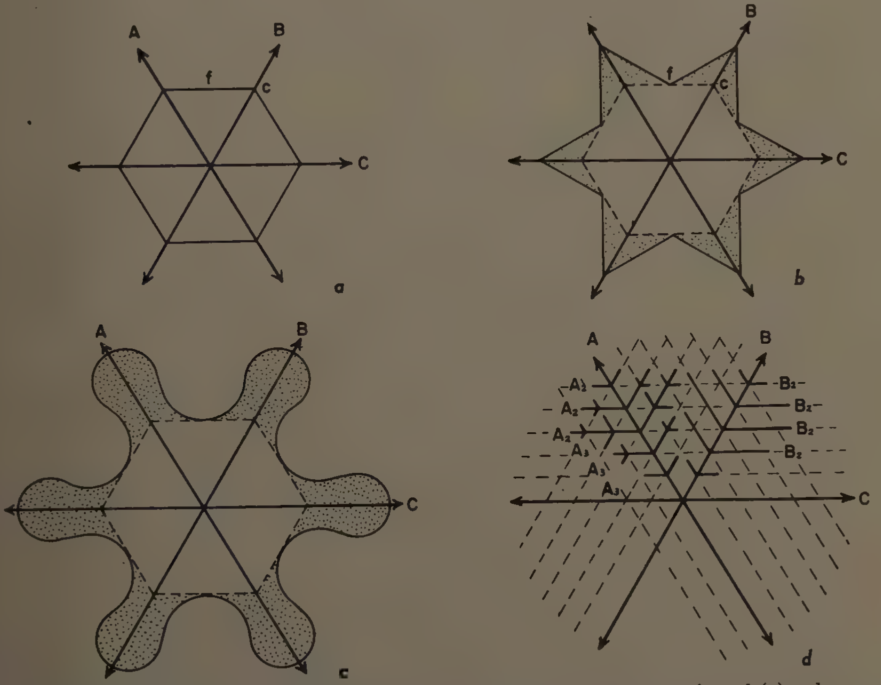

FIGURE 3. Diagrams illustrating the mechanism of the transformation of (*a*) a hexagonal plate into (*b*) a star, (*c*) a rosette, and (*d*) a skeleton. A, B, and C represent the 3 crystallographic axes perpendicular to the optical axis of the ice crystal; A_2, or B_2 and A_3 refer respectively to the secondary and the tertiary branches originating from the axes A and B; c (in diagram *a*) is one of the fast-growing corners; and f marks the center of a face of the hexagonal plate where growth ceases. Reproduced from Luyet and Rapatz, 1958, by permission of *Biodynamica.*

(B) MISCELLANEOUS TRANSITIONS

Solidification into amorphous ice. The possibility of obtaining water in the amorphous state has tempted several investigators (*cf.* Pryde and Jones, 1952). Of the various methods tried, the most reliable has been that in which molecules of water vapor are made to deposit on a cold plate in a vacuum (Burton and Oliver, 1935). The structure obtained could then be studied by X-ray diffraction, or the phase transitions could be detected by calorimetry. Both procedures were used (*see* review by Blackman and Lisgarten, 1958, p. 197, table designated as Figure 7), and both led to the conclusion that the material contained amorphous ice, but there were indications that the ice formed was only partially amorphous.

Glass transition. In the above-mentioned work, Pryde and Jones studied the occurrence, in amorphous ice, of the "glass transition," that is, of a transition characterized by a sudden increase in specific heat when the temperature is gradually raised but not involving any crystallization. In 1 experiment calorimetric determinations showed evidence of the glass transition between −125° and −150° C., but in subsequent experiments these authors were unable to confirm this observation.

Recrystallization. In the same experiments Pryde and Jones observed unexpectedly a heat-liberating phase transition at −129° C., in which the partly amorphous ice became crystalline. Contrary to the glass transition, this change was easily reproducible; it was, in fact, observed 29 times. Thus, a recrystallization upon warming of partly crystalline ice seems well established. (I designate this phenomenon as recrystallization rather than as devitrification to avoid the confusion resulting from the fact that a great multitude of structures is included under the term vitreous; *see* Luyet, 1957, pp. 439 to 441, and 1958, Section III).

Low-temperature biologists became interested in this transition and began to regard the temperature of −129° C. (or −130° C.) as a critical one in the study of solutions and of biological material. The idea generally held was that temperatures below −130° C. are safe for long-term preservation, as biological material would not undergo any phase transitions, whereas higher temperatures bring about instability and are therefore dangerous. Since this problem concerns solutions, it will be discussed below.

I may point out here some disagreement among investigators about the temperature of recrystallization of amorphous ice. According to the information tabulated by Blackman and Lisgarten (*l.c.*), none of the 5 groups of investigators who used the method of X-ray or electron diffraction recorded a passage from the amorphous to the crystalline state at −129° C., and 2 of the 4 investigators who used the calorimetric method agreed on that temperature, whereas the other 2 reported a transition some 12 to 15 degrees above it.

Recrystallization, as described, occurs rather abruptly at a given temperature, or in a rather narrow range. A gradual and slow recrystallization—sometimes designated as migratory recrystallization—may take place at various temperatures. Meryman (1957, Plate 19) has recorded it in electron micrographs, in the case of pure ice, at temperatures extending from −70° to −96° C.

Transition to cubic ice. Blackman's table, again, will be our guide to the literature on this subject. The 3 groups of workers who used electron-diffraction techniques reported cubic ice, 2 of them in a range of temperatures extending from about −150° to −80° C., the third between −120° and −100° C. Experimenters with calorimeters could not observe the transition in question, but their inability to do so could be attributed to the fact that too small amounts of heat were involved (Pryde and Jones, 1952).

Other transitions. I should mention here, finally, the transitions between some of the several types of crystalline ice obtained by Bridgman (1914) at high pressures.

(2) Phase Transitions in Solutions

(A) TYPICAL CRYSTALLIZATION

(a) *Primary Crystallization* (*Crystallization of Solvent in Relatively Dilute Solutions*)

Types of ice formations. Luyet and Rapatz (1958), in a study of the patterns of ice formation obtained with solutions of some 15 solutes, crystalloids and colloids, frozen at various cooling velocities and at various concentrations of the solutes, described 6 types of ice formation; these are represented in FIGURE 4.

Five of the types are obtained in the course of cooling, and the sixth is the result of a recrystallization obtained in rewarming; the latter is considered more in detail below under the heading MISCELLANEOUS TRANSITIONS. Of the 5 crystalline formations produced in cooling, one, the irregular rosette (FIGURE 4*d*) was obtained only in solutions of gelatin and of polyvinyl pyrrolidone. This formation apparently results from the particular entangling effect of long and stringy molecules. There remain 4 patterns (FIGURE 4): the regular polyhedra (*a*), the irregular dendrites (*b*), the coarse spherulites (*c*), and the evanescent spherulites (*e*), which seem to be controlled primarily by the cooling velocity, as we shall now see.

Fundamental effect of cooling velocity. When the cooling velocity is gradually increased one observes the passage from type 1 to types 2, 3, and 5 (represented, respectively, in *a*, *b*, *c*, and *e* of FIGURE 4). The regular polyhedra (*a*) are obtained in almost all solutions at high subzero temperatures. The reason is evidently that under these circumstances the molecules of water are given sufficient time to assume their orderly arrangement. At lower temperatures the regularity decreases (*b*); the number of radial columns originating from a center of crystallization is no longer limited to 6; it may be any number. However, remnants of the 60° angles between the columns and the lateral branches are still discernible. With higher cooling velocities one obtains spherulites (FIGURE 4*c*), consisting of innumerable radii that appear as thin, unbranched spears. When the cooling velocity is increased still further the spokes of the spherulites become so thin that they are no longer observable in ordinary light; in polarized light, however, between crossed Nicol prisms, the presence of the Maltese cross leaves no doubt about the crystallinity of the structure. There is evidence from X-ray analysis that crystallization of this type is incomplete (Meryman, 1958). It will be completed, upon a rise in temperature, in the process of recrystallization.

The effect of cooling velocity in bringing about those 4 types of crystalline structures is particularly well illustrated in the photographs of FIGURE 5, which were obtained in the freezing of 4 preparations of sucrose of the same concentration, cooled at different velocities in baths at different temperatures.

The evanescent spherulites and the recrystallization clouds need further attention but, since they represent more pronounced departures from typical

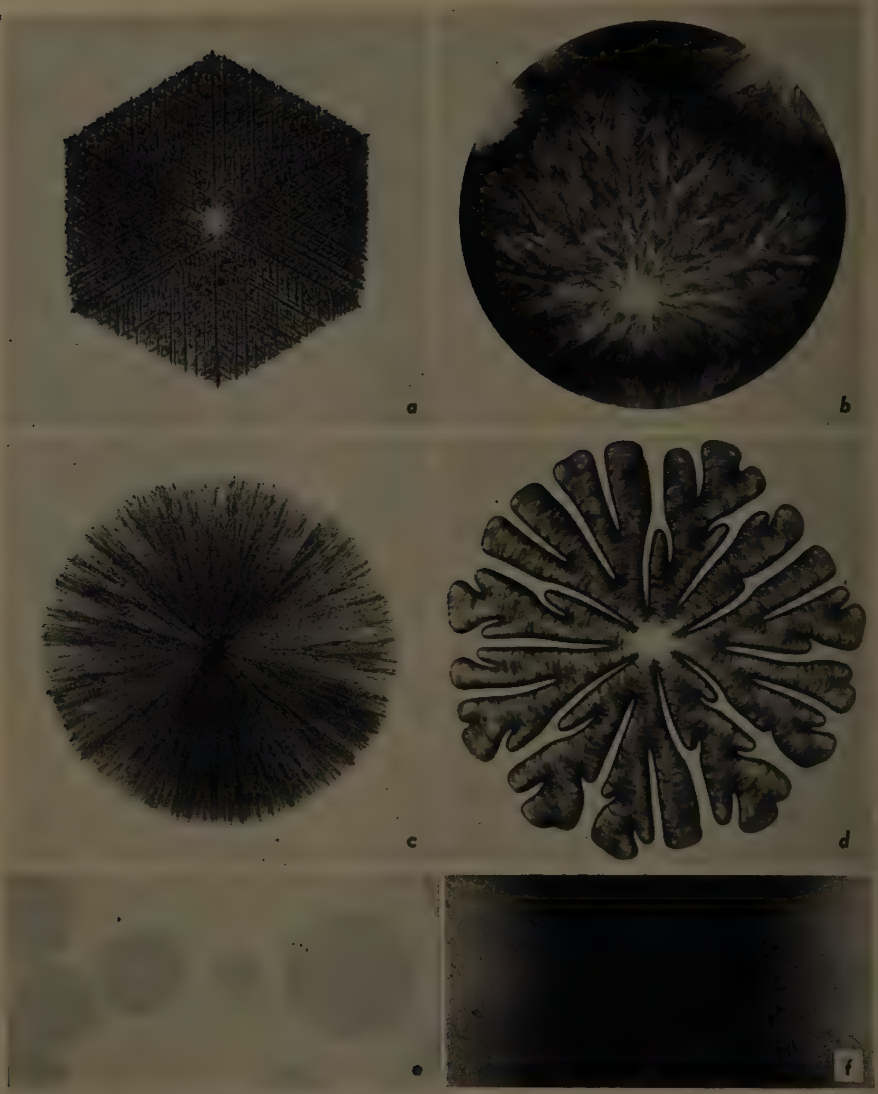

Figure 4. The 6 principal types of ice formations encountered in aqueous solutions at various solute concentrations and at various temperatures. (*a*) Hexagonal form obtained in 6M glycerol at −35° C.; (*b*) irregular dendrite in 10 per cent sucrose at −65° C.; (*c*) spherulite in 6 M glycerol at −60° C.; (*d*) lobed formation (or irregular rosette) in 50 per cent gelatin at −25° C.; (*e*) evanescent spherulite in 50 per cent sucrose at −45° C.; (*f*) recrystallization cloud in 30 per cent gelatin at −30° C. ×80. Reproduced from Luyet and Rapatz, 1958, by permission of *Biodynamica.*

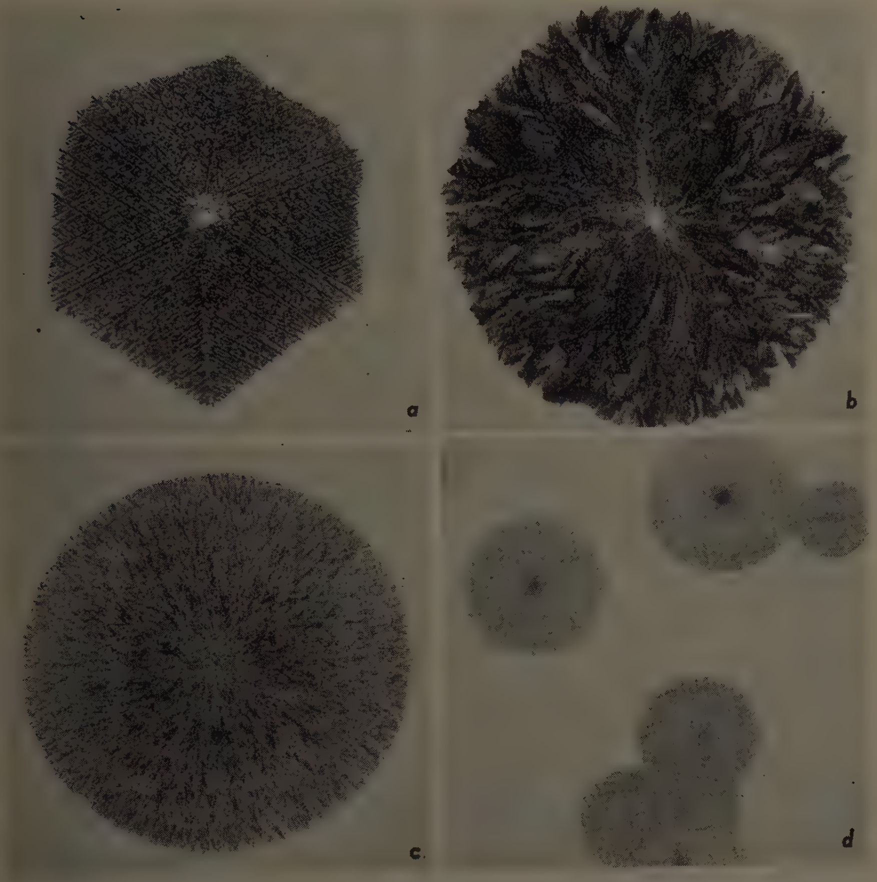

FIGURE 5. Effect of cooling velocity in causing the formation of (*a*) hexagonal structures; of spherulites with (*c*) coarse or (*d*) fine radii; and of (*b*) transition forms between hexagonal and spherulitic structures. The 4 types of crystalline formations shown were obtained in 50 per cent sucrose solutions when the bath temperature was, respectively (*a*) −20° C., (*b*) −25° C., (*c*) −30° C., and (*d*) −40° C. ×85. Reproduced from Luyet and Rapatz, 1958, by permission of *Biodynamica.*

crystallization, they will be discussed hereafter in the section MISCELLANEOUS TRANSITIONS.

(*b*) *Secondary Crystallization (Crystallizaton of Solvent and Solutes in Concentrated Solutions)*

Patterns of crystallization: (1) Method of study. To obtain preparations suitable for photography, that would show a primary crystallization resulting in an increase in solute concentration and followed by a secondary crystallization, we proceeded in the following manner. (I shall illustrate the procedure in the case of Ringer's solution, in which the main solute component is NaCl.)

First, we gradually lowered, from 0° to −20° C., the temperature of a thin layer of the solution placed between 2 cover glasses. During that cooling an increasing proportion of water crystallized out, forming arborescent structures, of which the parallel branches (column b in FIGURE 6*a*) were bathed in a pool of concentrated solution, seen in the channels c between the branches. We then lowered the temperature further (in this case, to −29° C.); the solution now crystallized out and appeared as a finely granulated mass, either in the channels c, between the columns b of ice, or above the columns, between them and the cover glass, in FIGURE 6*b*. Upon being rewarmed, the granulated mass melted at about −21° C. A different form of the same phenomenon is illustrated in FIGURE 6, *c* and *d* (*see* caption for details).

(2) Types of equilibria. The concentrated solution just described has all the characteristic properties of a eutectic solution. However, this is not necessarily what happens in other cases. The substances that crystallize out may be the solute itself or its hydrates, and not their mixture; if it is a mixture, it is not necessarily the eutectic mixture. I shall now consider briefly some other possible forms of equilibrium in the case of mixtures.

One of them, which may be of great importance in the freezing of biological material, is that of crystallization into solid solutions, also called mixed crystals. Hardy (1926) assumed their formation in the freezing of gelatin gels. However, our knowledge of the structure of solid solutions does not go far beyond simple cases in which the atoms of one component can be substituted for those of the other or be inserted between those of the other ("substitutional" and "interstitial" solid solutions); what happens in the freezing of solutions of large molecules such as those of proteins is still little known.

In regard to eutectic freezing (as defined in the classic cases of solutions of low-molecular-weight solutes), one should notice that it represents only one of many phase equilibria considered by physical chemists and metallurgists. Investigators enumerate, in binary systems only, as many as 26 possible types, of which 6 occur with sufficient frequency to be designated by special names: the eutectic, eutectoid, monotectic, peritectic, peritectoid, and syntectic types (Rhines, 1956, pages 100 and 101). Very few of them may occur in aqueous solutions, but some may be of significance. With systems of higher order—which will practically always be the rule in biological material—one will deal with ternary, quaternary, and higher eutectic freezings, possibly also with ternary, quaternary, and higher eutectoid, peritectic, and other phase transitions.

(3) Nonequilibrium conditions. Thus far only equilibrium conditions have been considered whereas, in most cases of freezing of biological material, crystallization proceeds too rapidly and the medium is too viscous to permit the establishment of an equilibrium. One would therefore expect all forms of hindered or incomplete secondary crystallization to be encountered. In the case of eutectic systems, one would expect a predominance of hypo- and hypereutectic crystallization, that is, with an excess of the ice phase or an excess of the solute phase, in the mixture.

(4) Types of crystallization units. One recognizes, in the freezing of concentrated solutions, the same patterns of crystallization as those reported in

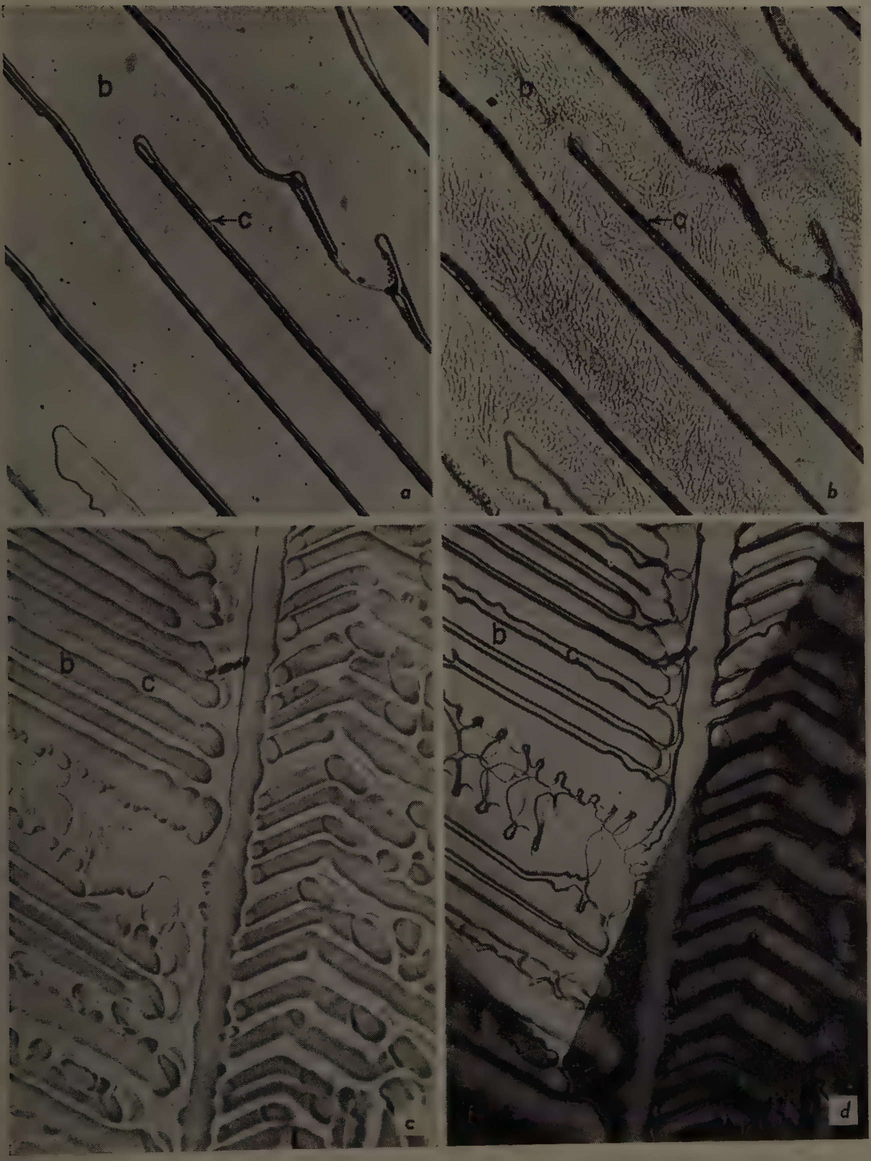

FIGURE 6. Primary and secondary crystallizations in Ringer's solution and in sodium chloride solutions. (*a*) Primary crystallization (crystallization of the solvent) in Ringer's solution at −21° C.; b in diagram *a*, branches of a dendrite of ice; c, in diagram *a*, channels containing the unfrozen, concentrated solution. (*b*) Secondary crystallization in the same solution at −29° C., same field as in *a* (the solution itself has now frozen in the channels c and at b, above the columns of ice). (*c*) Primary crystallization in a 15 per cent solution of NaCl at −21° C. (the channels c, between the columns b of ice, contain the unfrozen, concentrated solution). (*d*) Secondary crystallization in the same solution at −35° C. (same field as in *c*). Note the circular advancing fronts of 2 crystallization units (dark areas) which, developing from 2 centers outside the field, spread through the concentrated solution, between the columns of ice b, and above them. ×80. Reproduced from Luyet and Rapatz, in press, by permission of *Biodynamica*.

the freezing of the solvent in dilute solutions, that is, patterns of decreasing regularity, from the typical polyhedral forms to the irregular dendrites, then to the spherulites and, finally, to the evanescent spherulites. This is illustrated in FIGURES 7 and 8 (*see* captions for details).

Effect of cooling velocity. The 4 types of crystallization units just mentioned are obtained, in the order given, when one merely increases the cooling velocity; 3 of them are represented in FIGURE 8.

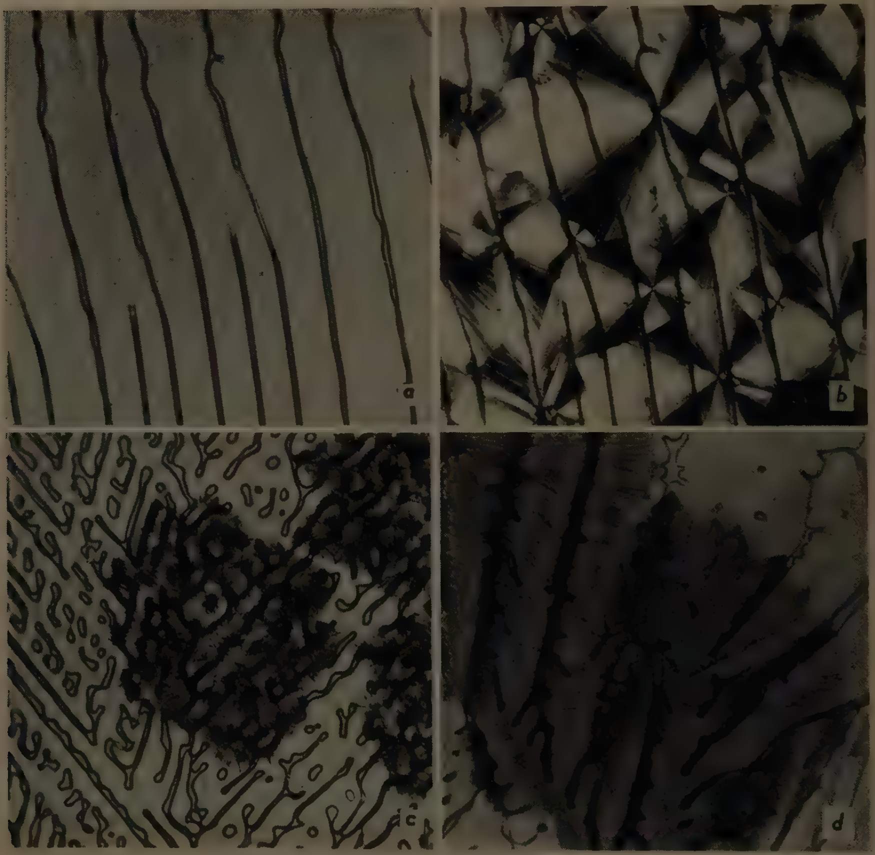

FIGURE 7. Primary and secondary crystallizations in solutions of glycine and beta-alanine. (*a*) Primary crystallization (crystallization of the solvent) in a 20 per cent solution of glycine at −4° C. (the white columns are ice; the narrow channels, between them, contain the unfrozen, concentrated solution). (*b*) Secondary crystallization of this concentrated solution into evanescent spherulites at −40° C.; same field as in *a*, but seen in polarized light between crossed Nicol prisms (the spherulites are superimposed on a background of ice columns). (*c*) Secondary crystallization into polyhedra (dark formations) in a 20 per cent solution of beta-alanine at −18° C. (the polyhedral structures are developing above the dendritic framework of ice formed in the primary crystallization and in the channels between the masses of ice). (*d*) Secondary crystallization into irregular dendrites (dark formations) in a 20 per cent solution of beta-alanine at −30° C. (the 2 units, developing from 2 centers of crystallization, are spreading above the dendritic framework of ice formed in the primary crystallization and in the channels between the masses of ice). ×81. Reproduced from Luyet and Rapatz, in press, by permission of *Biodynamica*.

(B) Miscellaneous Transitions

Solidification into the amorphous state. From the viewpoint of the biological applications, to solidify the material without permitting crystallization (that is, without disturbing the molecular structure) is perhaps the most important goal. I have discussed elsewhere the problem of the probable state of the so-called vitreous material (Luyet, 1957, pp. 439 to 441), and that of the practical impossibility of obtaining complete "vitrification" (Luyet, 1958). It will suffice here to restate the essential conclusions: vitreous material apparently consists of an ordered phase (crystallites) randomly dispersed in a nonordered medium; the ordered phase may be typical polyhedra, filaments, layers, bundles, or other aggregates of molecules or atoms. Under those conditions what one accomplishes in cooling rapidly, or in adding solutes, or in dehydrating is generally to bring about some intermediate state of incomplete crystallization.

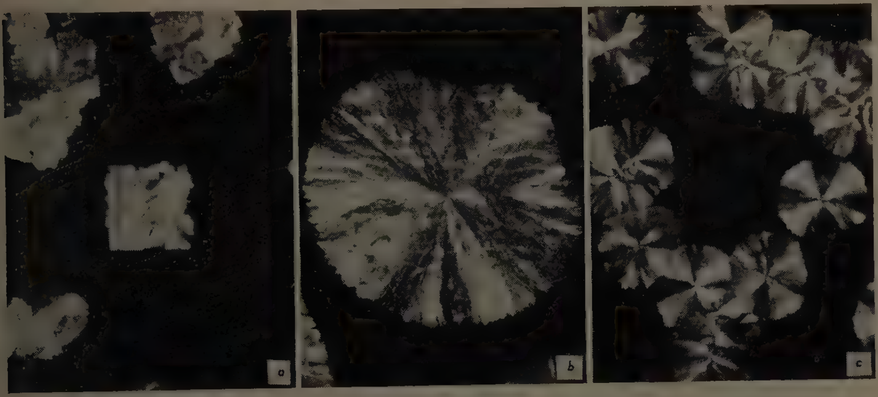

FIGURE 8. Effect of cooling velocity in causing the formation (*a*) of polyhedral structures or of spherulites with (*b*) coarse or (*c*) fine radii in a secondary crystallization of a solution of beta-alanine. The 3 types of crystalline formations shown were obtained in a 20 per cent solution when the bath temperature was, respectively (*a*) −20° C.; (*b*) −47° C.; and (*c*) −65° C. ×49. Reproduced from Luyet and Rapatz, in press, by permission of *Biodynamica.*

In the particular case of gelatin gels, for example, it seems that a completely amorphous state (as amorphous as in a liquid, that is, with only short-range order) is obtained only with gels of concentrations higher than 65 per cent. With solutions containing from 65 to 20 per cent gelatin we showed that the material often designated as vitreous is cryptocrystalline (Luyet and Rapatz, 1957 and 1958); and, as already mentioned, Meryman (1958) has given evidence, from X-ray diffraction, that it may be in some form of incomplete crystallization.

To sum up the situation in regard to the amorphous state of protoplasm after freezing, it seems that our present methods for rapid cooling can hinder the crystallization process somewhat, but do not prevent it altogether.

Recrystallization. Here I use the term recrystallization to designate a crystallization that takes place in the course of the warming of frozen solutions. This has been observed to happen in the following two sets of circumstances. (1)

When a preparation (consisting of a thin layer of solution) has been cooled rapidly, so as to remain transparent, and is rewarmed rather rapidly, it becomes quite suddenly opaque, and this change takes place within a relatively narrow range of temperatures; (2) when a similar preparation has been cooled slowly, so as to contain crystals sufficiently large to be seen under the microscope and is rewarmed slowly, the large crystals grow slowly larger while the small ones disappear, and this change takes place within a broad range of temperatures. Since, in the first case, crystalline particles become suddenly visible, I propose to call the change irruptive recrystallization (formerly it was often called devitrification). In the second case the change has been named migratory recrystallization, as it involves a migration of molecules from the small to the large crystals.

It is not implied here that the processes described are of fundamentally different nature in the two cases; the main differences between them may reside in the circumstances in which they occur, such as rapid versus slow cooling, rapid versus slow rewarming, and growth from small crystallites or nuclei versus growth from relatively large crystals.

(1) Irruptive recrystallization. This phenomenon can well be demonstrated with a 30 per cent gelatin gel. A thin layer of such gel is cooled rapidly by immersion into liquid nitrogen; it solidifies in a transparent mass that in ordinary light may not show any evident crystalline structure, either to the naked eye or under a microscope. However, in polarized light it has the properties of a spherulite, as described above and as illustrated in FIGURE 9*b* (the control is shown in FIGURE 9*a*). Then, upon a rise in temperature, at about −10° C. the material becomes opaque as a result of the growth of its crystallites or nuclei. The phenomenon is a "recrystallization," that is, a resumption of a previously initiated crystallization. FIGURE 9*c* and *d* shows an evanescent spherulite before and after recrystallization.

An interesting characteristic of irruptive recrystallization is that it occurs in a range of temperatures that is hardly affected by the concentration of the solute, but is greatly affected by its molecular weight. Thus, it varies only from −31.8° C. for a 2-M solution of sucrose to −31.4° C. for a 1-M solution (Luyet, 1939), whereas it varies from −12.6° C. for 45 per cent gelatin (Gehenio and Luyet, in press) to about −65° C. for 45 per cent glycerol (Luyet, 1941). These values are for the recrystallization, in 5 min., of a layer 0.1 mm. thick into an intensely opaque mass; traces of incipient opacity were sometimes observed some 10 to 15 degrees lower (Gehenio and Luyet), although their further intensification could not be ascertained in much longer times. The position of the range in terms of the size of the molecules of the solute is represented in FIGURE 10.

The release of heat during the irruptive recrystallization of glycerol solutions has been reported by Luyet *et al.* (1958). Their data are plotted in FIGURE 11.

It seems quite certain that the transition reported by Pryde and Jones (1952) to occur at −129° C. in pure water is of the same nature as that which we observed from about −80° C. in formaldehyde solutions to −6° C. in soluble starch. Thus, to free the molecules of water from their partly amorphous, disordered surroundings and make them available to join growing ice crystals would require a rise in temperature to only −129° C. if the surrounding consists of pure water to some −90° C., if it consists of a solution of very small molecules, and to near zero if it consists of a solution of very large molecules.

Consequently, the assumption that the temperature of −129° C., observed by Pryde and Jones for the recrystallization of pure water, is a critical temperature above which any frozen solution is unstable does not seem justified.

(2) Migratory recrystallization. The increase in size of large crystals at the expense of small ones, in frozen solutions, can easily be studied with the apparatus designed by Rapatz and Luyet (1957) for photographing thin preparations in the course of freezing. I reproduce here some photographs excerpted from a study of this phenomenon now in progress by these authors (FIGURE 12).

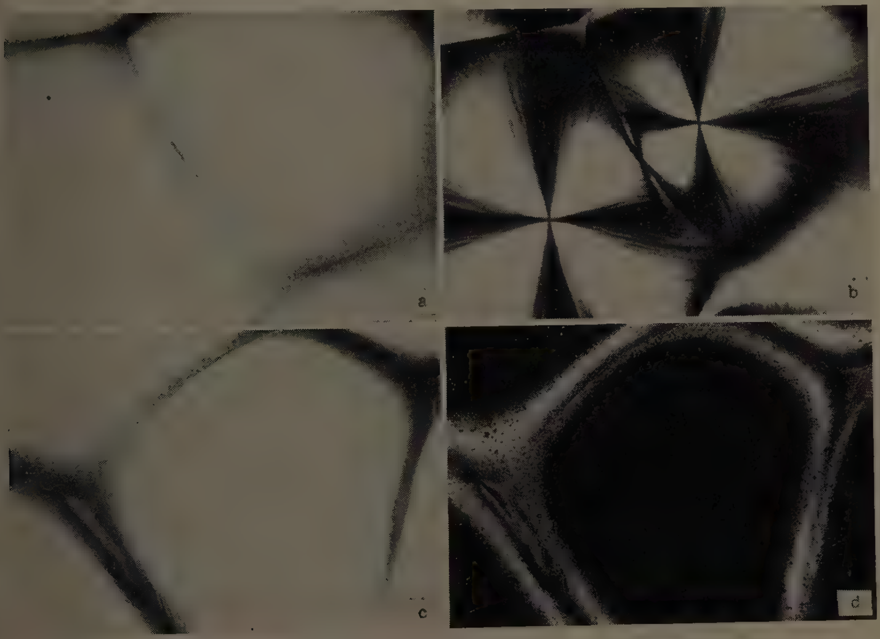

FIGURE 9. Evanescent spherulites in 30 per cent gelatin gels before and after recrystallization. (*a*) Gel frozen at −40° C., showing 2 evanescent spherulites (transparent areas) under ordinary light before recrystallization. (*b*) Same field under polarized light between crossed Nicol prisms before recrystallization. (*c*) Gel frozen at −35° C., showing 1 evanescent spherulite under ordinary light before recrystallization. (*d*) The same gel after recrystallization upon rewarming to −10° C. ×59. Reproduced from Rapatz and Luyet, in press, by permission of *Biodynamica*.

FIGURE 12*a* and *b* shows the changes that take place in a 10 per cent solution of glycerol maintained at −20° C. for 15 min. FIGURE 12*c* and *d* shows the absence of change in a 10 per cent gelatin gel maintained at −20° C. for 36 hours.

According to the information available thus far in that work, frozen solutions readily undergo migratory recrystallization at and above the temperature of irruptive recrystallization, and are stable for quite long periods of time below that temperature. However, why anticipate the final results? The true story will be told by the quantitative data on temperatures and times of recrystallization, which are forthcoming. (I remind the reader that the temperatures of irruptive recrystallization given in FIGURE 10 are for recrystallization

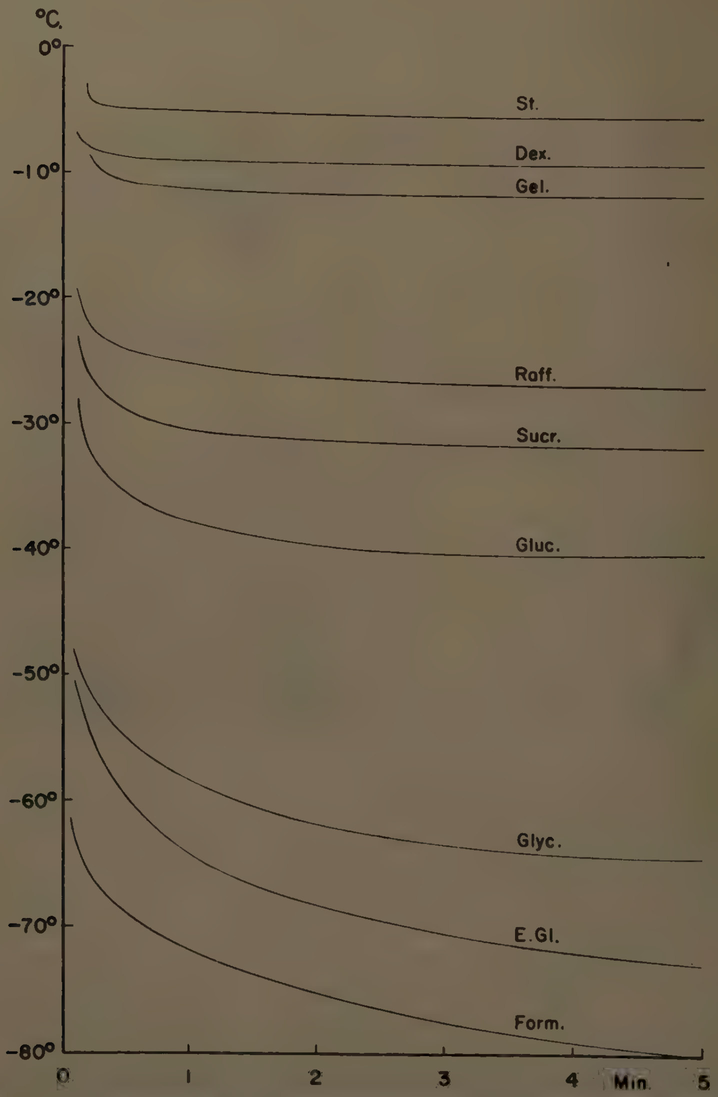

FIGURE 10. Temperatures of irruptive recrystallization in solutions of soluble starch (St.), dextrin (Dex.), gelatin (Gel.), raffinose (Raff.), sucrose (Sucr.), glucose (Gluc.), glycerol (Glyc.), ethylene glycol (E. Gl.), and formaldehyde (Form.). The concentrations used (which have only a slight effect on temperatures) varied from about 30 to 50 per cent by weight. The times plotted are those required for the recrystallizing specimens to become entirely opaque at the temperatures indicated in abscissa. Excerpted mainly from Luyet (1939 and 1941).

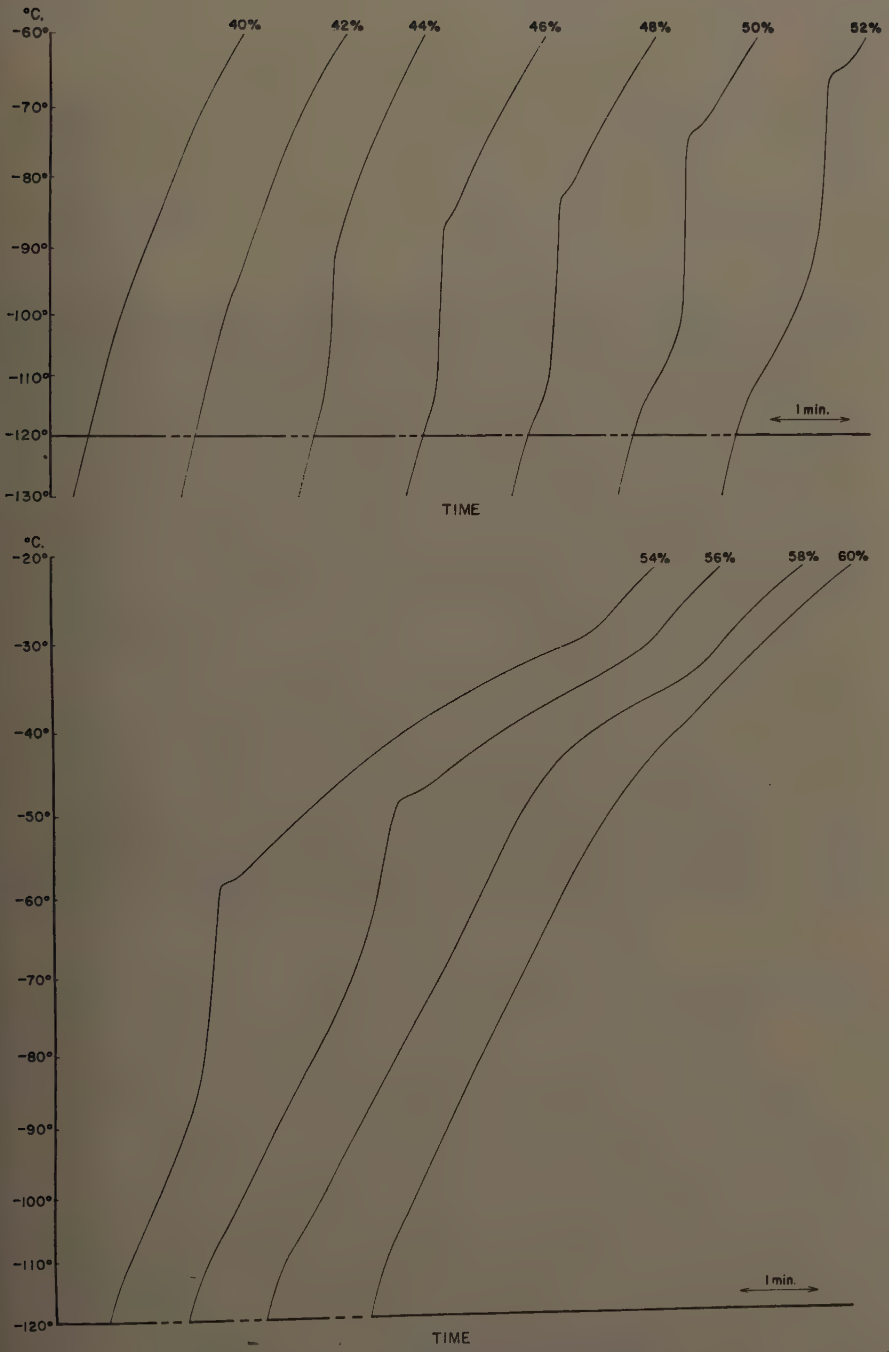

FIGURE 11. Heat release during irruptive recrystallization. Rewarming curves of glycerol-water mixtures of the composition indicated after rapid cooling to −195° C. The curves obtained with concentrations from 42 to 60 per cent show a rapid rise in temperature when the mixture undergoes irruptive recrystallization. For each curve the time is to be counted at the scale shown on the right side of the graphs, from the point of intersection of the curve with the abscissa, as the origin. Reproduced from Luyet *et al.* (1958) by permission of *Biodynamica.*

into an intensely opaque mass in 5 min. and cannot be taken as the lowest temperatures at which all traces of recrystallization are excluded in any period of time.)

Glass transition. The results obtained by Luyet and Kroener (1958) in an attempt to detect, in rewarming curves, the temperature of the glass transition of glycerol-water solutions of concentrations varying from 96.4 to 58 per cent are as follows:

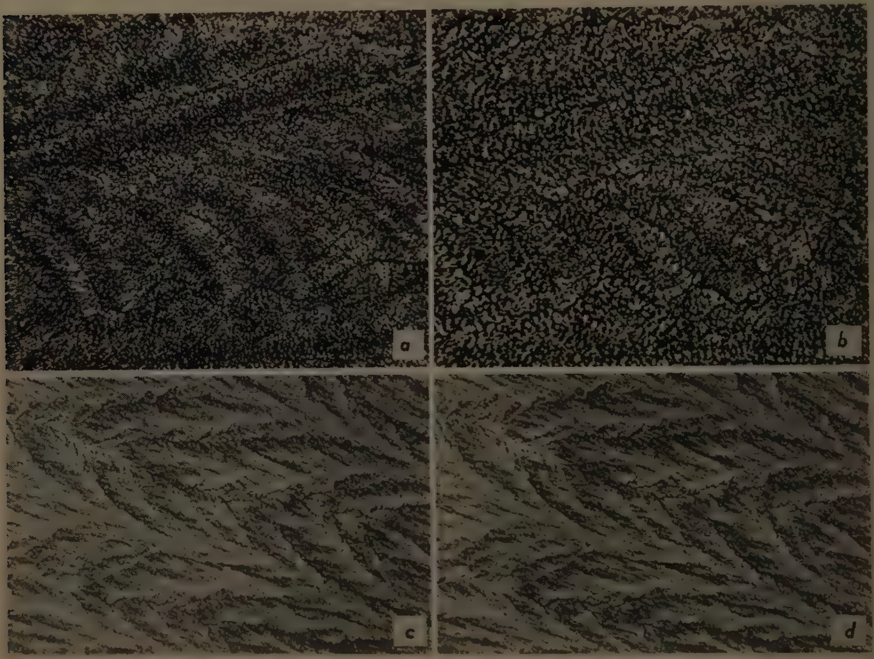

FIGURE 12. Migratory recrystallization. (*a* and *c*) Frozen solutions of, respectively, 10 per cent glycerol and 10 per cent gelatin before recrystallization; (*b*) shows the changes that had taken place in the field represented in *a* after 15 min. at −20° C.; (*d*) shows that no change had taken place in the field represented in *c* after 36 hours at −20° C. ×75. Reproduced from Rapatz and Luyet, in preparation.

Concentration:	96.4%	90%	80%	70%	60%	58%
Temperature:	−86° C.	−92° C.	−100° C.	−106° C.	−111° C.	−112° C.

The warming curves are plotted in FIGURE 13. It is interesting to note that extrapolation of the curve of decreasing temperatures at increasing water contents may lead to values of the order found by Pryde and Jones (1952) for the glass transition in amorphous pure water (from −125° to −150° C.).

Complete surveys of phase transitions in solutions. For the biologist interested in the stability of frozen solutions at various low temperatures, the ideal would be that he could chart all the phase transitions that take place in those solutions within given ranges of concentrations and temperatures. In an attempt in that direction, in the case of glycerol solutions I reported, on the graph of FIGURE

14, the transitions now known, that is, a primary crystallization (line AE), a secondary crystallization (line EB)—including a eutectic crystallization (point E)—an irruptive recrystallization (line R), and a glass transition (line G). Migratory recrystallization of ice crystals will probably take place in the area located between the line R and the line AE (within the reservations made above).

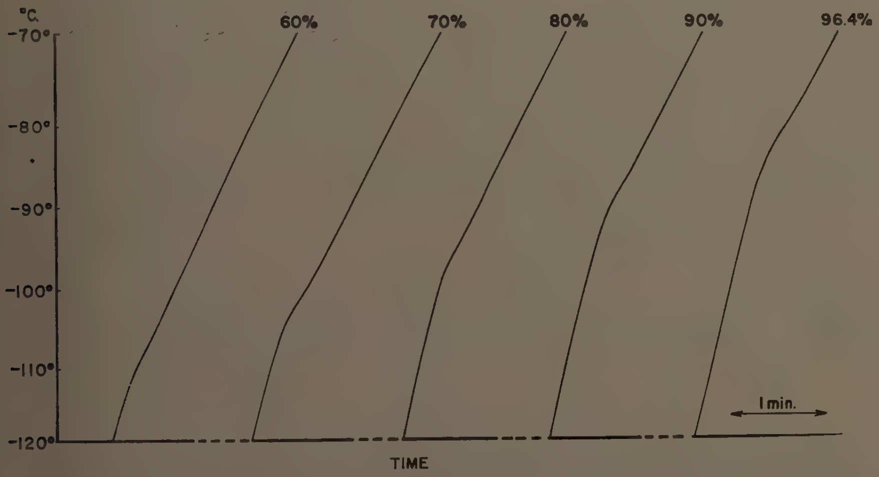

FIGURE 13. Rewarming curves showing the temperatures at which the glass transition takes place (sudden changes in slopes of the curves) in glycerol-water mixtures of various concentrations. For each curve the time is to be counted at the scale shown on the right side of the graphs, from the point of intersection of the curve with the abscissa, as the origin. Reproduced from Luyet and Kroener, in press, by permission of *Biodynamica*.

General Conspectus and Conclusions

Great variety of semicrystalline structures. From the relatively perfect structural order represented by ice crystals formed slowly in pure water or in most solutions to the disorder (long-range disorder) that prevails in water and in solutions in the liquid state, there is a whole world of intermediate structures. They constitute a magnificent array of patterns of disorder, varying both in the degree of disorder and in the proportion of material in disorder.

Intricacies of the crystallization process. The transition from the disordered to the ordered state is claimed to be often an intricate and laborious process. Thus, in many cases, if not generally, crystallization would be accomplished in two steps, the first consisting of the formation of crystallites or small aggregates of molecules (polyhedra, plates, spirals, bundles, filaments, and so on), and the second of an ordering of these aggregates. The large number of possibilities, both in the mode of arrangement and in the degree of order reached in each of the two steps, would give rise to an endless variety of developmental processes. In other cases, in which long-chain molecules are present, as in plastics, crystallization may consist in the tying of the neighbor chains at an increasing number of points along the chains, the resultant effect, at the macroscopic scale, being the types of plastic designated as leathery, rubbery, and so on. The variety of developmental processes that keeps the plastic industry and art in business is also one of the inspirational motives in the ceramic, glass, and metal industries and arts. These arts are really built on a ground of molecular disorder.

One may notice also that, whereas perfect crystallization consists in the

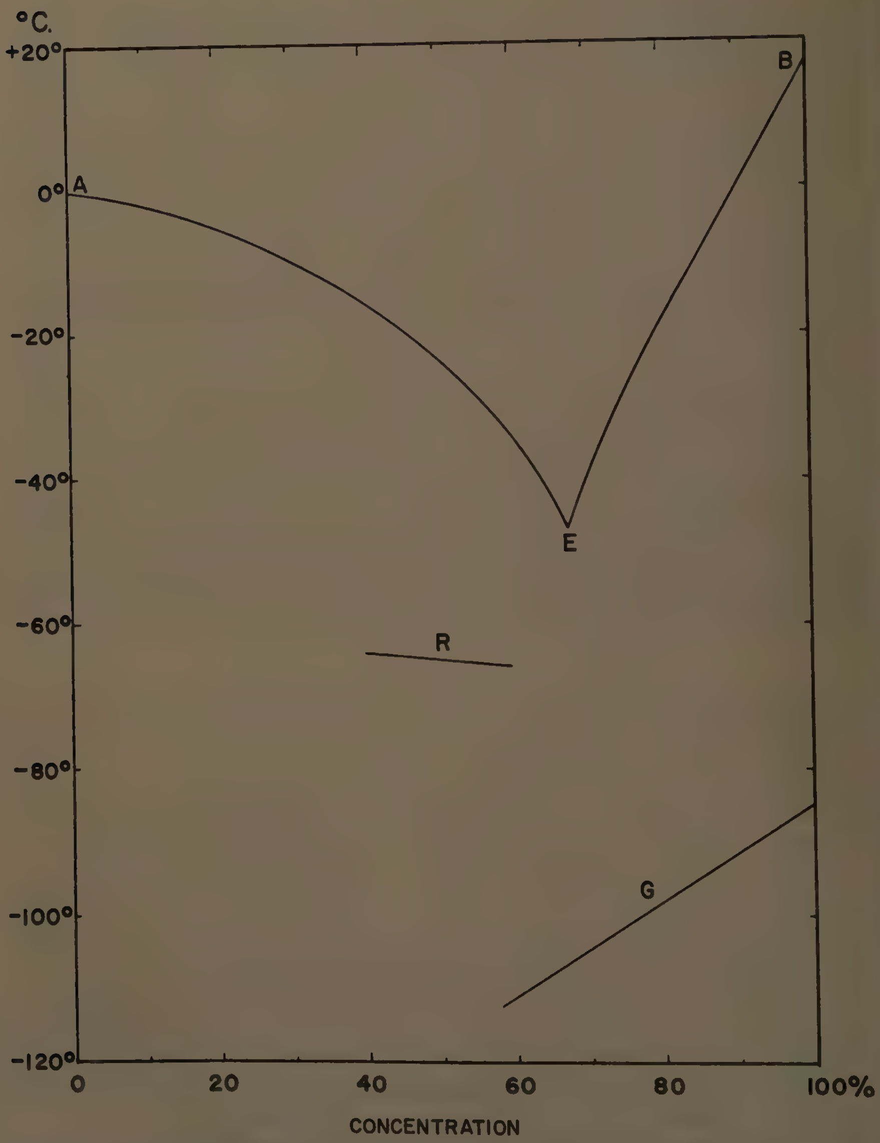

FIGURE 14. Temperatures and concentrations at which phase transitions are known to occur in glycerol-water mixtures: ordinate, temperature; abscissa, concentration of glycerol; AE, primary crystallization; E, eutectic point; EB, secondary crystallization; R, irruptive recrystallization; and G, glass transition.

attainment of an equilibrium, matter in the process of crystallization is on its way toward structural perfection and equilibrium. In the study of the freezing of solutions, we are therefore dealing with a world of complex structures on the move toward equilibrium, but hindered in the pursuit of their aim by a horde of obstacles.

A unifying principle. The biologist thus finds himself in a chaos of disequilibrium and disorder; but this situation, in a sense, suits him, for he wishes to avoid the order brought about by crystallization. On the other hand, to control the factors that hinder crystallization he must study them; that is, he must analyze the elements of the chaos. In that colossal work he has a unifying principle; it is the cooling velocity that controls the degree of order and of equilibrium attained in all forms of crystallization, as has been emphasized throughout this paper. The science, art, and industry of freezing biological material thus appears to consist primarily in the knowledge and the control of the cooling velocity.

Acknowledgments

All the photographs reproduced in this paper were taken by my research associate G. Rapatz; the drawings are the work of M. Persidsky. I express to them here my gratitude and appreciation.

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Part II. Freezing of Living Cells

FREEZING INJURY OF PLANT TISSUE

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About 50 years ago the eminent Russian plant physiologist N. A. Maximov summarized the problem of freezing injury in plants in the following colorful prose:

"Seldom does the opportunity occur to observe such pronounced changes in nature as after a sudden first frost. Plants that were previously bursting with life now offer a picture of complete destruction; everywhere are leaves and young shoots limp and blackened as though burnt by the frost. Tender shoots lie on the ground robbed of their turgor. Flowers have become wilted, their lively colors replaced by brown and dirty shades. Within this melancholy picture, there stand out plants that are better able to resist the frost. Not only may they fail to show any injury, but they may actually continue to bloom and produce new shoots until the falling snow hides them from the eyes. This perennially repeated picture gives rise to the following questions: Why is the frost destructive? How can some plants be more resistant than others though they differ from the others in no obvious manner?"[1]

To these two questions posed by Maximov, I add a third: "What is the moment of injury?" The following is a brief summary of what is known today about these three questions.

The Moment of Injury

Some of the early investigators believed that ice formation could occur only in dead plant tissues, and that only those that could prevent this ice formation survived. This was soon disproved by cutting into tissues during freezing weather. Not only could the ice be detected by the hardness of the tissue, but it could actually be seen. These same tissues were frequently found to be completely uninjured after thawing. Now that it was known that plants could survive freezing, the next question was whether the injury occurred during freezing or thawing. Evidence was obtained for both explanations by determining the effect of rates of freezing and thawing on the injury. Slow freezing quite generally reduced the amount of injury. Slow thawing did so also, but far less frequently. More direct evidence of injury during freezing was produced by the development of post-mortem colors and odors in tissues still in the frozen state. The amount of injury may also increase somewhat with the length of time the specimen has been frozen (after equilibrium has been reached). Finally, it has been shown also that the survival of the tissues sometimes depends on the postthawing treatment. There are therefore actually four possible moments of injury: (1) during the freezing process, (2) while frozen but after equilibrium has been reached, (3) during thawing, and (4) after thawing. It is obvious, of course, that killing during any one moment precludes any injury during the succeeding moments. Thus there can be no effect of rate of thawing if the plant has already been killed by the freezing.

The terms slow and rapid are, of course, relative, and may mean different things to different persons. In laboratory experiments under the microscope slow freezing may mean a drop of not more than 1 or 2° C./min., and rapid freezing a drop of 5 to 20° C./min. Temperature drops of 100° C./min. or more might perhaps be termed ultrarapid. In nature and in experimental freezing of plants in freezing chambers, slow freezing may mean not more than 1 or 2° C./hour. It is possible to combine slow with ultrarapid rates of freezing; thus Sakai[2] was able to plunge mulberry twigs into liquid N_2 and keep them there for 60 days without injury, provided that the first stage of freezing down to −30° C. was slow.

The Mechanism of Injury

Direct observation of tissues by many investigators revealed two kinds of freezing: (1) intracellular, and (2) extracellular. Intracellular freezing is always or nearly always fatal (except, of course, when ultrarapid; *see* Luyet, elsewhere in this monograph). Furthermore, only extracellular freezing has been observed under natural conditions. However, it has been possible to observe intracellular ice formation in whole plants frozen artificially under conditions that conceivably could occur in nature. It is therefore possible that, at least in some cases, the greater injury resulting from rapid freezing in nature is due to intracellular ice formation. One such possibility is a kind of injury known as sunscald, which occurs on the south side of trees in the Northern Hemisphere. The tissues on this side may thaw on a bright winter day if unprotected from the sun. If the shade temperature is sufficiently low, such a tree may refreeze after sunset with such rapidity as to permit the formation of intracellular ice and consequent death.

This, however, is theory; it remains a fact that under natural conditions only extracellular ice formation has been observed. Only the injury caused by this type of freezing will, therefore, be considered. The early theories can be quickly dismissed. The first recorded was the caloric theory, according to which injury was due to a loss of heat, which was then considered to be a substance.[3] The discovery that tissues that could be killed by freezing were able to survive supercooling for indefinite periods destroyed the basis for this theory. The second was the rupture theory, according to which the formation of ice within the cells ruptured them, causing death.[4] This theory is still found in some relatively recent books, although it was discarded more than a century ago on the basis of observations of thousands of cells after freezing. No cell rupture was found. The principal observation considered in favor of the theory was the filling of the intracellular spaces with water on thawing. However, this water is reabsorbed by uninjured tissues, so it cannot have leaked out of the cells as a result of their rupture. As further proof, it was shown that plant tissues contract rather than expand on freezing. Now that we know ice formation is normally extracellular, this theory is solely of historical interest.

Nearly eighty years ago a more promising theory was proposed, namely, the protein precipitation theory.[5,6] According to this concept, ice formation concentrated the cell solutes until a point was reached at which the salts or acids were sufficiently concentrated to precipitate or denature the proteins, resulting in death of the cell. This theory also was soon discarded for several reasons.

The most decisive evidence against it was produced by Maximov shortly after the theory was proposed. Maximov found that the ability of cells to survive freezing was greatly increased by the simple expedient of freezing them in solutions of nonpenetrating and nontoxic substances. These results were corroborated by several investigators, and the Swedish worker Å. Åkerman[8] proved that the protection was associated with plasmolysis. It was quite obvious from these results that the cells could survive much more severe freezing and therefore a much greater concentration of their contents, provided that they were permitted to plasmolyze during freezing. Thus the injury could not be ascribed to a specific concentration of salts or acids, or even of the proteins themselves, and the protein precipitation theory was discarded. Recently, some German investigators have attempted to revive this concept, although they have made no attempt to disprove the above incontrovertible evidence.

The theory that has been most successful in explaining the known facts is Iljin's[9] mechanical theory. According to this concept, when ice forms extracellularly the contracting cell wall subjects the protoplasm to stresses and strains that may lead to mechanical injury. That artificially applied pressures can cause injury to protoplasm has, of course, long been known. The prevention of injury by plasmolysis is readily explained by this theory, since the release of the protoplasm from the cell wall prevents the development of any such stresses and strains. It also agrees with many other known facts, such as the general correlation between small cell size and ability to survive freezing and the greater resistance to applied pressures shown by frost-resistant protoplasm.

Prevention of Injury

Elsewhere in this monograph Luyet has shown how freezing injury can be prevented artificially. However, this is very different from the natural ability of the plant to prevent injury, that is, so-called frost resistance or frost hardiness. In the case of perennial overwintering plants, this ability rises to a peak during the winter and drops to a minimum during the spring. Furthermore, when different species and even different varieties of a species are compared, wide differences in the peak attained can be found. In fact, there are all gradations between those plants that can never develop any ability to survive freezing and those that can survive the lowest temperatures ever observed in nature. This variation gives ample opportunity to determine what factors are correlated with frost resistance. Two main kinds of factors have been established: an increase in sugar content and a change in the physical properties of protoplasm. The second factor cannot be specifically narrowed down as yet to one fundamental change. Among these alterations are an increase in permeability to polar substances, a decrease in the rigidity of the protoplasm on dehydration, and an increased ability to survive sudden deplasmolysis. More recently it has been possible to show that the chloroplasts develop an ability to bind more water when they become more frost-resistant.

It is therefore obvious that there is a minimum of two factors involved in frost resistance: one vacuolar, the other protoplasmic. The roles that these factors play can be explained best on the basis of Iljin's mechanical theory of frost injury.[6]

The above brief summary of freezing injury to plants is adequate to suggest that a great deal more is known about the effects of freezing in the case of plant than of animal cells. However, does this necessarily mean that the information obtained with plant cells may be applied directly to an understanding of the effects of freezing on animals? Let us consider a typical plant cell, which differs from animal cells in the three following principal respects:

(1) The greater part of the cell is occupied by a vacuole that is responsible for the frequently very high water content of plant tissues. In this vacuole are accumulated the sugars that play such an important role in the frost resistance of many plants.

(2) A relatively thick and rigid cell wall surrounds the protoplasm. This wall adheres to the protoplasm and is pulled in with it on extracellular ice formation. On rapid remoistening it snaps back to its original position before the protoplasm can do so and may possibly tear the protoplasmic surface in the process. Whatever wall the animal cell may possess is quite different both in chemical and physical properties from those of the plant cell wall. As a result, in general, animal cells do not plasmolyze and therefore cannot suffer the deplasmolysis injury described above.

(3) The plant cell is in contact with the air of the intercellular spaces, in contrast to many animal cells that are bathed in fluid.

In spite of these differences, there is one very decided similarity between plant and animal cells: the nature of the protoplasm itself. Recent electron micrographs have shown that plant protoplasm is even more similar to animal protoplasm than previously suspected, since it contains both the endoplasmic reticulum and the Golgi apparatus. Thus, all of the structural components of protoplasm are similar in plant and animal cells. Furthermore, if ice forms outside animal cells, their protoplasm may just as conceivably be exposed to stresses and strains as in the case of plant cells. It is therefore possible that the basic theory that has proved so successful in explaining frost injury of plant cells may apply to animal cells as well, although some features of the injury may differ, and some of the methods of combatting the injury that are present in plant cells may perhaps not be available to animal cells.

A Unified Theory of Freezing Injury

In view of the above similarities and differences between plant and animal cells, the question is whether a simple theory of freezing injury is applicable to all organisms. It has been suggested from time to time that perhaps there is no such thing as extracellular freezing injury. Perhaps the injury always occurs only as a result of a small amount of intracellular ice that is not detectable. Thus the role of the increase in cell permeability that accompanies an increase in frost resistance can be explained as follows. When ice forms extracellularly, the cell sap becomes concentrated, and its freezing point drops. If the rate of extracellular freezing keeps up with the rate of temperature drop, the freezing point of the cell sap will at all times be sufficiently low to prevent the formation of intracellular ice, since the plasma membrane protects it from inoculation by the external ice crystals, and the minute drop of cell sap is certain to supercool at least a fraction of a degree. However, if the protoplasm is not sufficiently permeable to water, the water molecules cannot diffuse from the cell sap to the external ice loci with sufficient rapidity to keep pace with the drop in temperature. The concentration of cell sap will then be cooled sufficiently below its freezing point to cause intracellular freezing and injury. The role of high cell permeability in frost-resistant cells would then be to prevent intracellular freezing injury, and direct tests have shown that intracellular freezing occurs more readily in nonresistant than in frost-resistant cells. However, it could not affect extracellular freezing injury if this phenomenon really occurs, and the evidence indicates conclusively that it does occur. Aside from the many cases of direct observation (and if the ice crystals inside the cell are too small to be seen, they are also probably too small to injure), it was long ago found possible to cut open a frozen potato tuber, remove the relatively few large ice crystals (each crystal having a volume equal to many dozens or hundreds of cells), and to show that the remaining tissues were soft and quite obviously unfrozen. On warming the potato the tissues were found to be killed. The most conclusive evidence, however, is the fact that frost resistance, drought resistance, and plasmolysis resistance are all correlated.[6,7] This has been shown to be true in a very large number of investigations conducted in different ways. In a few cases it has been possible even to obtain quantitative results and, whenever this has been done, the agreement is within the limits of error of the methods used. In other words, the injury is caused by the same degree of dehydration whether the water is removed in the form of vapor, liquid, or solid. It is thus obvious that these cells must be injured by the dehydration, no matter where the ice forms. Consequently, extracellular ice formation must be injurious if it results in a sufficient degree of dehydration. Any unified theory of freezing injury must therefore include both extracellular and intracellular freezing injury. The following theory is proposed:

Freezing injury is due in all cases to breaks in protoplasmic bonds as a result of local stresses arising on ice formation in the tissues. The stresses may arise in the following three ways:

(1) By the direct pressure due to ice formation. This is relatively rare and probably occurs mainly under artificial conditions, as when cells are frozen in an aqueous medium confined within the walls of a rigid container.

(2) By ice formed inside the cells. This intracellular freezing always results in injury provided that the ice crystals are sufficiently large and numerous to lacerate the protoplasm.

(3) By the collapse and subsequent expansion of the cells due to the dehydration accompanying extracellular ice formation and the rehydration on thawing.

The first process (pressure) would explain such injuries as occur in liquid cultures of microorganisms. The second (formation of intracellular ice) may be the sole cause of injury in the case of cells below a certain critical water content and/or size; that is, all those that are uninjured by dehydration. These cells are uninjured by extracellular freezing because the maximum dehydration fails to set up appreciable stresses. The third process (collapse and subsequent expansion) may occur only in the case of cells above a certain critical water content and/or size, including all or nearly all the mature cells of higher plants and perhaps many animal cells.

On the basis of this theory only cells injured by the third process can be protected by nonpenetrating solutions. Cells injured by the second process can be protected only by penetrating solutions. The assumption of a physical break of the protoplasmic bonds in both plant and animal cells is in agreement with the basic similarity in the physicochemical properties of the protoplasm of both kinds of cells. The assumption of a difference in the manner in which the stresses may be initiated is based on the differences in the other characteristics of plant and animal cells.

It should be emphasized that cells injured by the third process may, under other circumstances (such as rapid freezing) be injured by the second process and, at least under artificial conditions, also by the first process.

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FACTORS AFFECTING THE ERYTHROCYTE DURING RAPID FREEZING AND THAWING

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In recent years reports by Meryman and Kafig[1] and by Strumia *et al.*[2] have stimulated interest in the possibility of preserving blood by subjecting it to a rapid freezing process. The cooling of red cells to temperatures below their freezing point and their subsequent return to the liquid state is known to be an effective procedure for inducing hemolysis. The chain of events leading to hemolysis has been considered in some detail by Lovelock,[3,4] whose primary conclusion that salt concentration, brought about during the freezing process, is capable of modifying the red cell membrane provided a rationale for the use of high concentrations of glycerol as a protective additive in the low-temperature processing of blood. By holding water in the liquid state, thereby keeping electrolytes in solution until a freezing temperature is achieved at which salt-lipoprotein interactions proceed at a very slow rate, glycerol circumvents the processes that, during slow cooling and warming, normally lead to hemolysis. At glycerol concentrations of 40 per cent or more, freezing, in the sense of ice crystal formation, may not occur at all.

It is evident, however, that the success of the freeze-thaw experiments of Meryman and Kafig and of Strumia *et al.*, as well as unreported work in our laboratory in which additives other than glycerol were used, depends on rapid cooling and warming. The additives and concentrations used by these investigators, about 5 per cent glucose (by Meryman and Kafig) and 5 per cent glucose-7.5 per cent lactose (by Strumia *et al.*), limit the degree of hemolysis following thawing if due regard is paid the rate at which heat is removed from and supplied to the specimen. Their techniques of freezing and thawing are such as to limit the time spent by the cells at temperatures at which destructive reactions can proceed at appreciable rates. The additives provide protection over a critical temperature range, from the freezing point down to about −50° C. Thus, the rate at which heat can be transferred from and to the specimens and the time spent in temperature regions in which degradative reactions can proceed may be regarded as primary factors affecting the erythrocyte during rapid freezing and thawing. These factors are subject to control by varying the geometry of a specimen, the temperature differential in cooling and warming operations, and the various coefficients of heat transfer involved in a given system.

We have investigated the influence of these and other variables on red cell recovery in freeze-thaw processing. It has been the purpose of one aspect of our work to provide quantitative data by which the conditions necessary to achieve a high order of cell recovery, that is, 80 per cent or more, can be defined. Our experimental approach to the problem and discussion of the data obtained form the basis of this communication.

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Specimen Geometry and Heat Transfer

Aqueous systems, of which biological materials form a large group, are relatively poor conductors of heat. As a consequence, once the cooling operation has begun, a temperature gradient will be created in the specimen. If the temperature differential between the specimen and the heat sink is large, the gradient will be greater than if the differential were relatively small. The situation becomes more complex as the periphery of the specimen freezes, since the thermal conductivity and specific heat of the ice formed will differ markedly from the liquid in the interior. In cases of rapid, unsteady-state heat transfer the latter property may be of greater significance than the former. Under such conditions, however, the geometry of the specimen will determine the relative importance of each in the heat transfer process.

In many experimental problems rapid cooling may be not only advantageous but necessary. Among these may be included the freezing of various blood preparations. The thermal aspects of such problems are essentially those of unsteady-state heat transfer. The reduction in heat content of an aqueous system may be regarded as a three-step process. Heat is first removed from liquid; the latent heat of fusion, evolved during the phase change, follows; finally, the heat content of the solid phase is reduced. With a given cooling procedure the rates at which heat can be transferred from the specimen to the cooling agent will be a function of the specimen geometry. This is illustrated in FIGURES 1 through 4 in which three simple geometries are considered.

It will be useful to consider as a model system a cooling droplet of water. In treating the problem mathematically as a variation of the classic Stefan's problem[5] the following assumptions were made: no convection exists within the droplet; heat transfer is effected with complete spherical symmetry; and the heat-transfer coefficient through the vapor film between droplet and cooling medium is constant over the temperature range considered. For simplicity, solutions involving supercooling will not be considered here. The numerical solutions to the partial differential equations involved, obtained by the Datatron computer, are presented in graphic form in FIGURES 1 and 2.

FIGURE 1 shows a temperature-distance plot computed, in this case, for a water droplet 1 mm. in diameter. Each of the curves is isochronous. The droplet is assumed to be cooling in liquid nitrogen from an initial temperature of 20° C. Since it is impossible to use thermocouples to obtain temperature records in small droplets, the heat-transfer coefficient across the vapor film between the droplet surface and the liquid nitrogen reservoir was calculated to be 61×10^{-4} cal./sec. cm.^{2} °C. It will be seen that the curves give an instantaneous temperature profile across the radius.

If we view the droplet as a volume in which red blood cells are being cooled, this figure suggests the variation in the thermal histories of red cells located in different regions. For example, the maximum temperature gradient computed across the droplet radius under these conditions is between 20° and 25° C., occurring at 1.33 sec., despite the initial temperature differential between droplet and refrigerant of 216° C. Thus, the cells in the outer zones of a droplet do not cool instantaneously to the temperature of the refrigerant, a situation that would imply an infinitely high heat transfer coefficient, while the cells in the inner regions cool more slowly. Cells located in the 3 outermost zones would require about 0.7 to 1 sec. to pass through the temperature range 0° to $-20°$ C. The cells in the 3 innermost zones would remain in this temperature region for a period of about 0.02 sec., roughly one thirty-fifth to one-fiftieth the time spent by the cells at the peripheral zones. It will be seen that, at certain periods in the cooling process, the cells in the inner regions of the droplet would lose heat far more rapidly than the cells of the outer regions of the droplet.

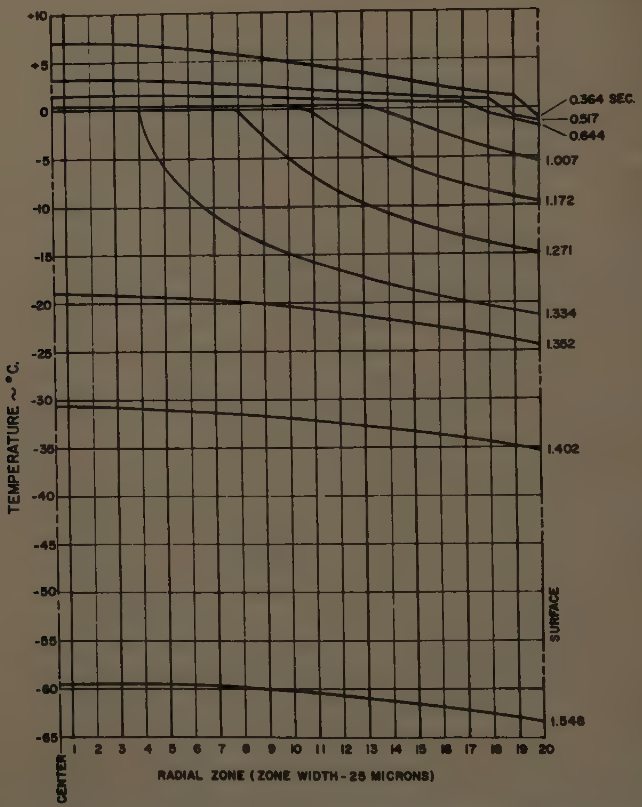

FIGURE 1. Thermal profiles computed for a droplet of water (0.5 mm. in radius) cooling in liquid nitrogen. Each ordinate marks the outer limit of a radial zone 25 μ in width.

Another aspect of the thermal history of cells cooling in a droplet under these conditions is found by examining the advance of the freezing front through the droplet.

FIGURE 2 shows a plot of the computed data for the inward progress of the liquid-solid interface with time. The nonlinearity of the curve shows clearly that the heat of fusion is removed at an ever-changing rate. For example, the region extending from about 100 to 150 μ from the center (ordinates 4 and 5) loses its latent heat about 8 times more rapidly than the region extending from

50 to 100 μ inward from the surface (ordinates 16 and 17). It is evident that the spherical geometry, by providing a decreasing liquid volume, requires that a decreasing amount of heat be removed per unit of radial distance during the freezing process.

Red cells frozen in droplet form in liquid nitrogen, with or without an additive such as glucose, show a high percentage recovery, as indicated by free hemoglobin levels, on thawing in a liquid medium such as plasma. Without additive we have obtained, in accord with the observations of Meryman,[1] recoveries of the order of 80 to 85 per cent. With a 0.3 M glucose additive we have routinely observed recoveries between 90 to 97 per cent. There are, of course, factors other than the technique of freezing involved in the recovery of cells containing intact complements of hemoglobin. In the case of the spherical geometry provided by the small droplet, however, mathematical analysis of the thermal conditions prevailing during cooling suggest that erythrocytes are capable of withstanding a rather broad range of heat removal rates, all of which are rather imprecisely referred to as "rapid."

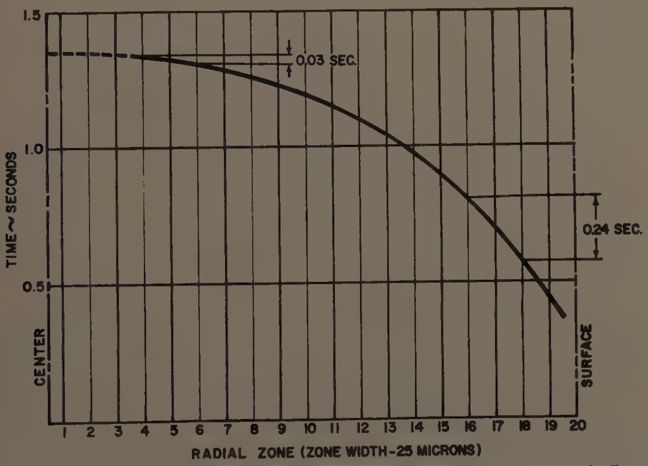

FIGURE 2. Advance of the ice-water interface computed for a droplet (0.5 mm. in radius) cooling in liquid nitrogen. Each ordinate marks the outer limit of a radial zone 25 μ in width.

Another simple geometry in which freeze-thaw operations with blood have been carried out with remarkable success by Strumia[2] is the rectangle with a thickness of a few millimeters. Oscilloscopic traces are reproduced in FIGURE 3 that were obtained in blood with 0.3 M lactose as a protective additive. Cooling was carried out in liquid nitrogen with an average heat transfer coefficient of about 61×10^{-4} cal./cm.^{2} sec. °C through the vapor film between container wall and refrigerant. The specimen was in a rectangular aluminum container 5 mm. in thickness (wall thickness, 0.5 mm.).

The 10-mil Chromel-constantan thermocouples, which provided the traces, were spaced as follows: the outer thermocouple was placed as near the container wall as conveniently possible without touching it, the next one was placed 1.2 mm. from the wall, and the innermost one was placed as closely as possible to the geometric center of the specimen. Hence, the traces are sensings of temperature obtained over a linear distance of about 2.5 mm. and are normal to the principal direction of heat flow.

The average rate of advance of the freezing front from the container wall to the center of the blood mass was slightly less than 0.3 mm./sec. The cells in the region closest to the container wall were in the temperature zone between the freezing point and −50° C. for about 5 sec. Those in the geometric center were in this zone for about 2.5 sec. Compared to the average values computed for a 1-mm. droplet (about 0.55 mm./sec.), the average rate of advance of the freezing front in this more massive geometry is considerably slower. As indicated by the position of the second trace, relative to the outer- and innermost traces, the latent heat is removed more rapidly as the ice boundary approaches the center of the cooling blood mass. In this respect the situation is qualitatively similar to that calculated to exist in the cooling droplet.

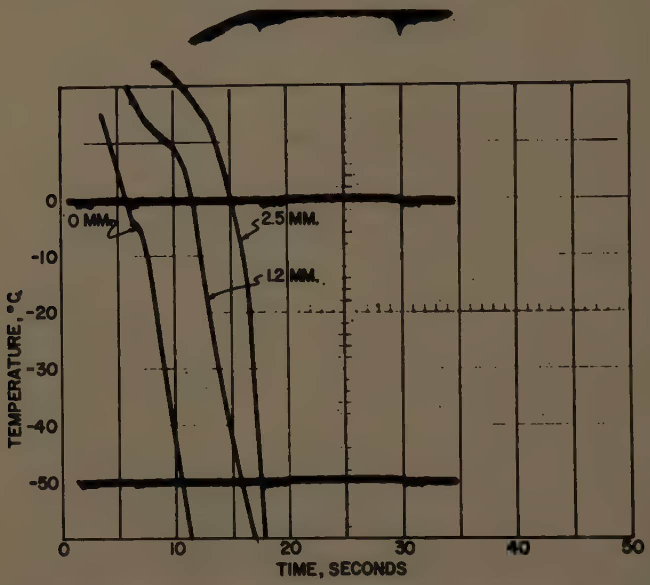

FIGURE 3. Oscilloscopic traces showing changes in temperature with time in a rectangular (5 mm. in thickness, 50 mm. in width) aluminum container of blood with 0.3 M lactose as a protective additive. Liquid nitrogen was the cooling agent; average outside heat transfer coefficient, 61 × 10^{-4} cal./cm.^{2} sec. °C. Thermocouples were placed as indicated, 0 mm. signifying that the outermost thermocouple was positioned as close as possible to the wall of the container without touching it.

In FIGURE 4 another simple geometry, that of the cylinder, is considered. The container, made of thin-walled (0.9 mm.) aluminum, was 11 mm. internal diameter. Ten-mil Chromel-constantan thermocouples were fixed near the wall, at the geometric center, and at a point between these. The relative position of the second thermocouple differed from that in the rectangular container discussed above, being somewhat closer to the center. Cooling was carried out in liquid nitrogen, the outside heat transfer coefficient being about 61 × 10^{-4} cal./cm.^{2} sec. ° C. The blood contained 0.3 M lactose.

A comparison of the time component in these traces with those obtained in the rectangular container is of some interest. Despite an increase in cross section of the cylinder more than twofold greater than the rectangle, the regions sensed by the thermocouples nearest the walls of both types of containers remained in the 0° to −50° C. zone for about the same length of time (approximately 5 sec.). The regions sensed by both thermocouples at the geometric centers also remained in this temperature zone for the same length of time (about 2.5 sec.). The thermocouples sensing the regions between wall and center, however, show clearly the influence of the cross section. Blood cells in the region 3.5 mm. from the outer wall of the cylinder remained in the temperature zone between 0° and −50° C. for 7 sec. In the rectangular container, at a distance of 1.2 mm. from the outer wall, they remained in this zone for about one-half that time (FIGURE 3).

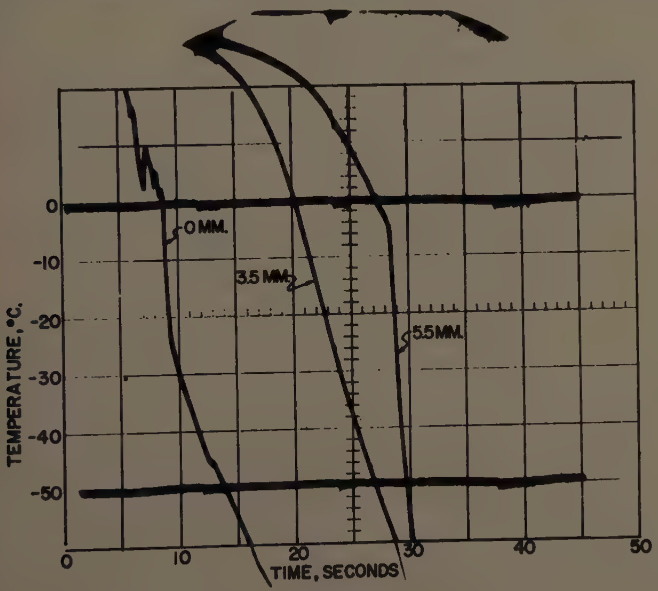

FIGURE 4. Oscilloscopic traces showing changes in temperature with time in a cylindrical (11 mm. in internal diameter) aluminum container of blood with 0.3 M lactose as a protective additive. Liquid nitrogen was the cooling agent; average outside heat transfer coefficient, 61 × 10^{-4} cal./cm.^{2} sec. °C. Thermocouples were placed as indicated, 0 mm. signifying that the outermost thermocouple was positioned as close as possible to the wall of the container without touching it.

In the cooling process, then, thermal analysis indicates that cells in the different regions of a specimen having a thickness of the order of 1 mm. or more undergo markedly different thermal histories. Such differences are pertinent to a time-temperature process, the purpose of which is to obtain as many intact cells as possible at its conclusion.

Warming a frozen volume of blood (the reverse of the cooling process) presents difficulties of considerable technical interest. The physics of the problem remain those of heat transfer, but with inherent limitations imposed by the thermal stability of the proteins present. To examine but one aspect: if liquid nitrogen were used as a refrigerant, then, during the freezing step, at the temperature at which ice begins to form, a temperature differential of more than 190° C. exists. Similarly, if the refrigerant were cooled to the temperature of solid CO_2, a differential of about 79° C. would be found as freezing began. The temperature differential can be regarded as a measure, in part, of the ability of the cooling agent to withdraw heat from the specimen. If, during the warming stage, the same differentials between the warming medium and the specimen were applied at the melting point, the results could be disastrous to cells and plasma proteins before the phase change was complete. In practice, then, during thawing we are restricted to a rather limited temperature differential at just that temperature region when the need for a rapid heat transfer is greatest from the viewpoint of the well-being of the cells.

TABLE 1

THAWING TIME FOR BLOOD DROPLETS IMMERSED IN STATIONARY BATHS

Temperature of thawing medium (°C.)	Diameter of droplet, mm. (closest estimate)	Time for complete thawing (sec.)
25	1.1	4.52
35	1.0	0.65
45	1.1	0.58
55	1.1	0.42

Thawing medium: 7 per cent polyvinylpyrrolidone in 0.85 per cent saline.
Temperature of droplet before entry to thawing bath was about −196° C.

TABLE 1 provides information on the significance of the temperature differential in thawing. The data were obtained by allowing single droplets at −196° C. to fall into stationary thawing baths maintained at various temperatures. The bath container was constructed of transparent plastic. The process was followed by high-speed cinematography, the droplet as it entered the bath being silhouetted against a calibrated grid. The end point of the process was taken as the change in density, resulting from the phase transition, which permitted the blood to start to sink in the thawing medium.

Brief mention of the events that attend thawing by immersion in an aqueous medium may be useful at this time, because they apply generally, regardless of geometry. When an object at a temperature considerably below the freezing point of ice is immersed in an aqueous thaw bath, the surrounding medium may be cooled to a point at which ice will form on the warming object. The lower the initial temperatures of the bath and the object being warmed, the greater the amount of ice that will form. In the case of the droplet immersed in a stationary medium, its diameter will be increased by ice formation. In addition, the surrounding fluid having cooled, the temperature differential (that is, the driving force in the immediate environment of the droplet tending to supply heat to it) will have decreased. The effects of additional ice formation and the lower temperature differential are thus compounded. The large increase in thawing time (about sevenfold) observed when the bath temperature is lowered from 35° to 25° C. illustrates the point (TABLE 1).

Were the bath maintained at the melting point of blood, the time required to thaw the droplet would be indefinitely prolonged. It is thus evident that a progressive lowering of the bath temperature below 25° C. will result in an extremely rapid increase in the amount of time required to effect thawing.

During freezing in liquid nitrogen the time required to take the droplet from 20° C. through completion of the phase change is about twice as long as that required to take the droplet from −196° C. through the phase change during thawing (in a stationary bath at 35° C. or higher). The slower transfer of heat while cooling is due to the formation of an insulating gas film as the droplet enters the liquid nitrogen. A thermal resistance is thus established in the cooling operation that does not exist in thawing. This is a point of considerable interest in experimentation designed to differentiate effects produced in cells or other materials of biological origin by differences in thermal histories during rapid cooling and warming. The spherical geometry and small diameter of the droplet permit the setting up of easily controlled conditions in which, despite a relatively low temperature differential during thawing, the residence time in a given temperature zone is less during warming than it is during the cooling operation.

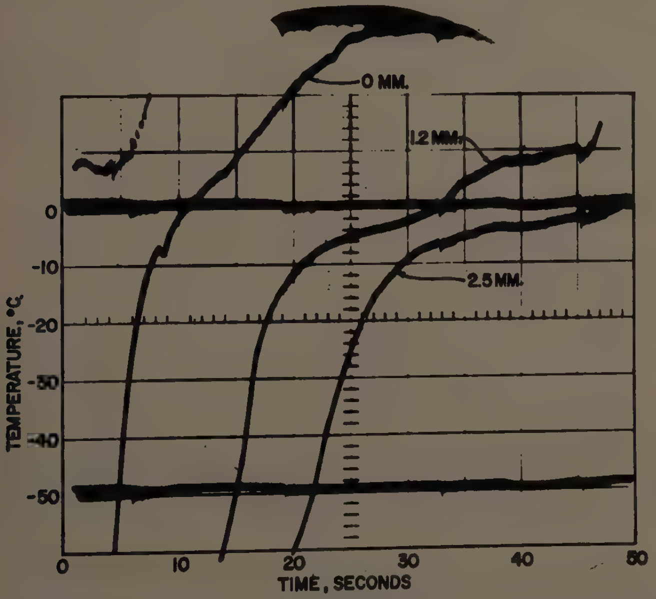

FIGURE 5. Oscilloscopic traces during warming, showing changes in temperature with time in a rectangular (5 mm. in thickness, 50 mm. in width) aluminum container of blood with 0.3 M lactose as a protective additive. The heat source was a static water bath at 45° C. Thermocouple placement was the same as that described in FIGURE 3.

FIGURE 5 shows the thermal traces obtained during warming of a blood-lactose (0.3 M) preparation in a rectangular aluminum container, 5 mm. in cross section. The bath was at 45° C. and static. The rapidly changing slopes of the traces indicate that a major problem during the thawing process is that of putting heat into the warming specimen over the temperature zone from −20° C. through the melting point. Unlike the droplet, the relatively massive geometry requires periods measured in tens of seconds to complete thawing. It is also apparent that the different shapes for each trace indicate distinctly different thermal histories for erythrocytes in different regions of the specimen.

Figure 6 shows warming traces for a blood-lactose (0.3 M) preparation in an aluminum cylinder 11 mm. in cross section. As in the preceding case, the bath was at 45° C. and static. Thermocouple placement was the same as that described for the studies on cooling (figure 4). The more massive geometry, relative to the 5-mm. container, is reflected in the rapidly changing curvature of the traces in the temperature zone between −30° C. and the melting point. The greatly increased time necessary to supply the latent heat of fusion is also evident in the prolonged horizontal slope of the inner traces.

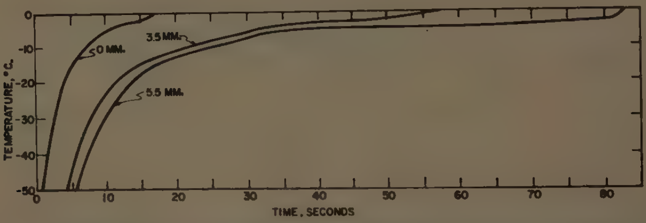

Figure 6. Oscilloscopic traces obtained during warming, showing changes in temperature with time in a cylindrical (11 mm. in internal diameter) aluminum container of blood with 0.3 M lactose as a protective additive. The heat source was a static water bath of 45° C. Thermocouple placement was the same as that indicated in figure 4.

Red Cell Recovery and Rapid Freeze-Thaw Procedures

The elementary considerations discussed thus far suggest that red cell recovery from so-called rapid freeze-thaw procedures may be regarded as an indication of the capacity of an average cell to withstand the physical and chemical consequences arising from widely differing thermal conditions. Without attempting to define an average red cell, it is probably reasonable to say that the distribution of the variables in an erythrocyte population, in experimentation of the type under discussion, will be uniform throughout the suspensions. If this is the case, an experimental approach that measures local hemolysis in regions that can be defined in terms of their thermal histories should permit closer determination of heat transfer rate limits that will yield high red cell recoveries. We are developing techniques to carry out such studies. These techniques appear to offer a fruitful method for assessing differences in red cell stability to regional differences in thermal history resulting from a freeze-thaw process.

Table 2 summarizes red cell recoveries as functions of gross differences in specimen geometry, and therefore of large over-all differences in thermal histories. Recovery was determined from measurements of percentage hemolysis using sample hematocrits and colorimetric determinations of supernatant and total hemoglobin. All samples consisted of blood and A.C.D. anticoagulant plus lactose at 0.3 M final concentration. Droplets were 1-mm. spherical particles obtained by spraying blood into liquid nitrogen. The heat transfer coefficient at the droplet surface was approximately 61 × 10^{-4} cal./cm.^{2} sec. °C. The droplets were thawed by stirring into plasma at 47° C. The remaining samples consisted of blood contained within thin-walled aluminum cylinders or tubes of rectangular cross section. Liquid nitrogen was the coolant, with an outside heat transfer coefficient in each case similar to that at the droplet surface, that is, about 61 × 10^{-4} cal./cm.^{2} sec. °C. Warming from −196° C. to the liquid state was done by immersion of the containers in a stationary bath of water at 45° C. With the red cell recovery in 1-mm. droplets as the reference standard, there has been roughly a 2 per cent decline in recovery for each millimeter of increase in specimen thickness under experimental conditions where the bulk specimens were processed while held stationary.

TABLE 2
RED CELL RECOVERY AND SAMPLE GEOMETRY

Sample geometry	Cross section (mm.)	Red cell recovery (%)
Spherical droplets (no container)	1	92
Cylinder	6	81.0 ± 3.3
Flat plate (rectangular cross section)	5	80.5 ± 3.5
Cylinder	11	66.5 ± 3.5

All samples consisted of blood containing 0.3 M lactose.

Specimens in rectangular and cylindrical containers were cooled and warmed while held stationary.

Cooling medium was liquid nitrogen (average heat transfer coefficient over temperature range 0 to −50° C. was 61 × 10^{-4} cal./sec. cm.^{2} °C.).

Warming medium was a water bath at 45° C. (average heat transfer coefficient over temperature range 0° to −50° C. was 108 × 10^{-4} cal./sec. cm.^{2} °C.).

Barriers to Heat Transfer

Certain aspects of the problem of heat transfer were noted earlier. It may be helpful to consider some of the physical detail involved. In a commonplace situation, such as one might encounter when cooling a fluid in a tube, there are four separate barriers through which heat must pass; these are diagramed in FIGURE 7. The data were taken from an actual experiment for which an instantaneous analysis has been made. A cylindrical aluminum container, with an internal diameter of 17 mm., containing serum was cooled with liquid nitrogen. The temperatures were continuously recorded by high-speed temperature recording equipment, one 10-mil thermocouple being placed at the center, another 5 mm. from the wall, and the third as close to the wall as possible without touching it.

For convenience the aluminum wall, indicated by the hatched lines in FIGURE 7, has been scaled in degrees centrigrade. A close approximation of the thermal profile existing in the system 1 min. after the cooling process has begun is represented by the angular line extending across the diagram. The heat barriers are the liquid layer, the ice layer, the container wall, and the gas film between the outer container wall and the coolant. The relative ease with which

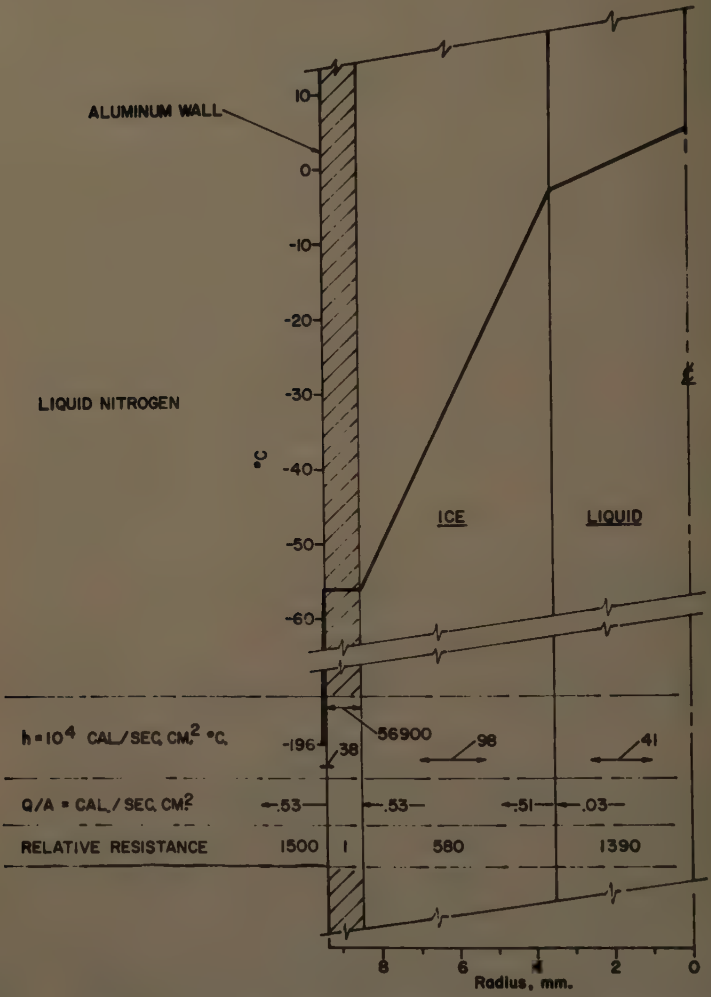

FIGURE 7. Diagram showing the instantaneous temperature profile in serum being cooled in an aluminum cylinder 17 mm. in diameter 60 sec. after cooling with liquid nitrogen was initiated. Key: *h*, heat transfer coefficient; *Q/A*, heat flux. The relative resistance is the reciprocal of *h* divided by that for the aluminum wall.

heat can be transferred through 3 of these—liquid, ice, and wall—can be evaluated by dividing the thermal conductivity of each by the length of the path through which heat must travel. These "conductance" or *h* values are shown in the first row of data at the bottom of the diagram.

A somewhat simpler view of the situation can be obtained by taking the reciprocal of these values and dividing it by that obtained for the aluminum wall. The relative resistance to heat transfer of each barrier with respect to the aluminum wall is thus obtained. These values are shown in the last row of data. The negligible resistance of the container wall is immediately apparent. Furthermore, under the experimental conditions used, at this instant in the cooling operation the values for the outside resistance and the liquid resistance are close, roughly 2.5 times greater than that for ice.

We have found the concept of thermal resistances (or conductances) useful in making experimental comparisons between different cooling and warming techniques in our work with blood. It is clear that a reduction in one resistance would lower the over-all resistance, that is, less time would be required to achieve a given temperature in a given region of the cooling specimen. However, the advantage gained by altering one resistance soon tends to plateau. For example, if we were to reduce the resistance at the container-coolant interface to one tenth of its value by pumping or pressure-spraying liquid nitrogen, there would be a reduction in over-all resistance of about 39 per cent. If we were able to reduce this resistance to one one-hundredth of its original value, we would gain only another 10 per cent.

Having considered the heat conductance or resistance of each portion of the system, it remains to examine the sources of heat. The latent heat of fusion is, of course, being elaborated at the ice-liquid interface and must pass through the ice. Thus, 0.03 cal./sec. cm.^{2} are leaving the body of the liquid, the source of which is the heat content of the fluid; but 0.51 cal./sec. cm.^{2} are entering the ice, as shown in the second row of data giving the heat fluxes. The difference between 0.03 and 0.51 is due to latent heat being generated at the interface. As indicated by the data in the second row at the bottom of FIGURE 7, an additional 0.02 cal. are entering and leaving the container wall, the source of which is the heat content of the solid phase.

Finally it will be seen that, under these experimental conditions, the freezing front has advanced 5 mm. in 60 sec. If the erythrocytes in a blood preparation cooled in this manner were oriented with their diameters lying in horizontal planes, cell edge touching cell edge, about 625 cells would have been included in the advance of the solid phase through any single plane 1 cell in thickness. This represents a minimum. The maximum (about 3300 cells) would be within the solid phase at this instant, if the orientation of the diameters were vertical and cell face touched cell face. The true number of cells included in the solid phase of a uniform suspension would lie somewhere in between these 2 figures. While the rate of advance of the freezing front is not constant, a rough calculation suggests that between 10 and 55 erythrocytes have been included in the ice phase (in a given horizontal plane of cellular dimensions) during each second of the cooling operation.

The thermal status of the same system discussed in FIGURE 7 is shown in FIGURE 8 during an instant in the warming process. The solid-liquid interface is the same distance, 5 mm., from the container wall, as was illustrated in FIGURE 7. The heavy sloping line extending across the diagram indicates the temperature profile, not at 60 sec. after the start of the heat transfer process as in cooling, but at 117 sec. Almost twice the time was required to put the liquid-solid interface 5 mm. from the container wall during the warming operation. While the same barriers to heat flow exist as in cooling, their relative resistances have changed. The dominance of the liquid layer is apparent in the values shown in the last row of data. Thus, if the resistance at the container-water bath interface were reduced to half its value, the over-all thermal resistance would be changed by only 8 per cent. If the liquid resistance could be reduced by one half, however, an over-all reduction of 40 per cent in total thermal resistance would be effected. This is a point of considerable importance in the rapid warming of red cells, because the latent heat must be supplied to the interface through the layer with the highest thermal resistance. This sug-

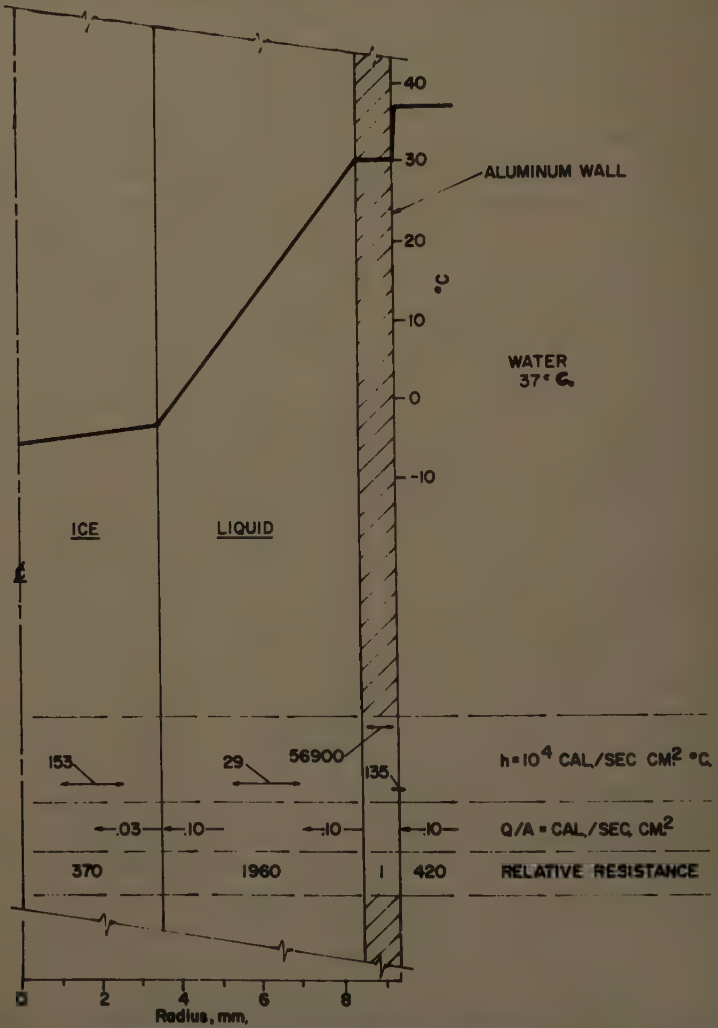

FIGURE 8. Diagram showing the instantaneous temperature profile in serum being warmed from −196° C. in an aluminum cylinder 17 mm. in diameter 117 sec. after warming was initiated in a water bath at 37° C. under static conditions. The symbols are the same as those used in FIGURE 7.

gests that forced convection through the liquid, such as might be achieved by shaking the warming container, would accelerate the thawing process. Although heat transfer at the ice-liquid interface would still be effected by conduction through an essentially stagnant liquid layer, the thickness of this layer would be greatly reduced relative to that in a motionless container.

Red Cell Recovery and Thermal Resistance

The effect on red cell recovery of varying thermal resistances during rapid cooling and warming is shown in TABLE 3. In this series of experiments the thickness of the blood mass, the outside heat transfer coefficient, and the resistance of the liquid phase inside the container have been varied.

Consider first the container with a blood mass thickness of 14 mm. Increasing the outside heat transfer coefficient from 41 to 61 × 10^{-4} cal./sec. cm.^{2} °C., a 50 per cent increase, produced a 50 per cent increase in red cell recovery (from 47 per cent to 70 per cent). From these and subsequent data in TABLE 3, it is apparent that in a "rapid-freeze" process the rates of temperature change *during cooling* can be of major importance to the integrity of the erythrocyte. In this system it is also evident that the outside coefficient at its lowest value (41 × 10^{-4}) is dominant in controlling the flow of heat during much of the cooling operation, since a major change in it is reflected in a major change in another heat-flow dependent parameter, in this case red cell recovery. A further increase of 77 per cent in the outside heat transfer coefficient of the 14-mm. container (from 61 to 108 × 10^{-4} cal./sec. cm.^{2} °C.) resulted in red cell recovery improved by only 15 per cent. The trend to a plateau mentioned earlier in connection with the continuing decrease of any one thermal resistance is seen here.

TABLE 3

FACTORS AFFECTING RED CELL RECOVERY DURING FREEZING AND THAWING

Blood mass thickness (mm.)	Additive concentration	Heat transfer coefficient during cooling (Cal./sec. cm.^{2} °C.)	Shaken during freezing	Shaken during thawing	Red cell recovery (%)
14	0.3 M lactose	41 × 10^{-4}	no	no	47
14	0.3 M lactose	61 × 10^{-4}	no	no	70
14	0.3 M lactose	108 × 10^{-4}	no	no	81
14	0.3 M lactose	61 × 10^{-4}	no	yes	85
17	0.15 M lactose	108 × 10^{-4}	no	yes	83
17	0.15 M lactose	108 × 10^{-4}	yes	yes	93

Freezing medium, liquid nitrogen (−196° C.); thawing medium, water (45° C.); container material, aluminum (0.9 mm.); geometry, cylindrical; blood volume, 14 mm. (15 cc.) and 17 mm. (21 cc.).

Thermal resistances other than those at the outer surface of the container can be of significance to red cell recovery. This is seen when we compare the same outside heat transfer coefficients (61 × 10^{-4} cal./sec. cm.^{2} °C.) during cooling, but reduce the resistance through the liquid layer by shaking one of the containers during warming. The reduction in thermal resistance (or, conversely, the improvement in the heat transfer coefficient) in the liquid layer gives nearly the same improvement in recovery of erythrocytes as does raising the outside coefficient from 61 to 108 $\times$ 10^{-4} cal./sec. cm.^{2} °C.

In the container with a blood mass thickness of 17 mm., a high outside heat transfer coefficient during cooling, combined with improvement of heat transfer in the liquid layer achieved by shaking during warming, gave almost the same red cell recovery as the 14-mm. system with the same outside heat transfer coefficient; that is, 83 and 81 per cent, respectively. From a practical point of view, however, it is significant that the reduced thermal resistances established in the 17-mm. system allowed a 50 per cent reduction in the amount of protective additive while yielding comparable erythrocyte recovery. Furthermore, when reduced thermal resistance in the liquid layer was effected by shaking during the cooling as well as the warming operation, the percentage of cells recovered from the process increased from 83 to 93. This latter observation emphasizes the importance of cooling rates, as distinct from warming, to red cell integrity in a technique designed to process blood rapidly.

Earlier, allusion was made to a critical temperature zone, with the implication that the time of residence by the frozen blood cells in this zone affected their stability on re-entering a liquid environment. It is not possible here to enter into an analysis of the factors that lend criticalness to this zone. For the present purpose it is defined simply as the temperature range between the freezing point and −50° C. As has been shown, the thermal histories of cells in different regions of a specimen will vary considerably. Despite this, in geometries of narrow cross section, less than 10 mm. for example, we have frequently found it useful to examine the temperature-time course in a single region and relate this to over-all red cell recovery on thawing.

FIGURE 9 presents curves in which recovery is plotted against the time spent in cooling the central region of the blood mass from 0° to −50° C. Times were obtained from traces provided by thermocouples centered in aluminum tubes of 6-mm. internal diameter. Lactose was used as a protective additive.

Since the time spent in the 0° to −50° C. zone is an indicator of the rates at which heat is being removed, the existence of maxima in the two curves show that cooling can take place too rapidly as well as too slowly. These data appear to confirm the earlier observations of Gehenio and Luyet[6] that cooling can be too rapid for the well-being of the erythrocyte. There is, however, another point brought out by the curves in FIGURE 9. Slower warming tends to enlarge differences in red cell recovery. In the preparations warmed in the 30° C. bath, those specimens taken through the 0° to −50° C. zone in 2 sec. or less average about 42 per cent fewer intact cells than those passing through this zone in 6 sec. In the more rapidly warmed preparations this difference is about 9 per cent. The material taken through the 0° to −50° C. zone in 11 sec. shows about 17 per cent fewer intact cells relative to the cells residing in the zone for 6 sec. when the slower warming procedure was used. The same comparison in the more rapid warming procedure shows a difference of about 11 per cent. Apparently, potentially destructive conditions created during cooling may actually become destructive only if the duration of the thawing process permits these conditions to become operative on the red cell. Thus, differences in recovery in the upper curve are much less than in the lower one. The data indicate the time dependence of unfavorable reactions or interactions in blood subjected to rapid freeze-thaw procedures.

FIGURE 10 presents data concerning the time of residence in a zone between −10° and −50° C. as it affects red cell recovery. Here the total time spent in this zone is examined, rather than, as in FIGURE 9, the time during the cooling phase only. A variety of geometries is represented in the different points. Blood mass thicknesses ranged from 4 to 17 mm., a thermocouple being placed at the center of each specimen. The time-temperature curves obtained during cooling and warming were used to calculate residence time of this central region in the −10° to −50° C. zone. While considerable scatter in the points is evident, it should be recalled that recovery values represent an average for all the regions in the blood specimen, while the time values are those obtained over very small volumes in the immediate vicinities of the centrally placed thermocouples. Despite the limitations of the experimental method a relationship between residence times in this temperature zone and red cell recovery is indicated.

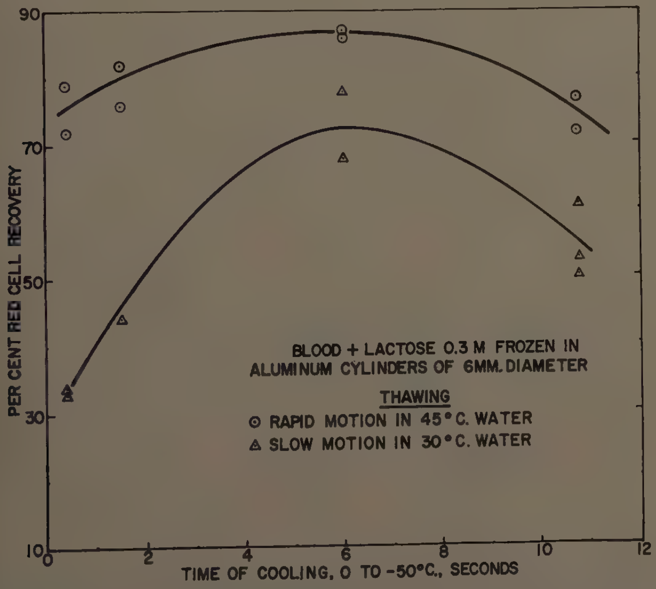

FIGURE 9. Plot of red cell recovery against the time of residence of the central regions of volumes of blood within the temperature range 0° to −50° C. during cooling.

The difference in protective effect between the lactose additive and the glucose-lactose combination is immediately evident. The latter mixture, first used by Strumia,[2] has been studied extensively by him and in our laboratory. The points shown by crosses were taken from the data presented by Strumia *et al.* in a recent publication of the International Institute of Refrigeration.[7] They are not strictly comparable, since they represent residence times in the zone between −3° and −40° C., rather than −10° and −50° C., as do all other points. They are shown here to illustrate the general agreement of results observed in experiments employing similar additives and cooling rates.

Protective Additives and Heat Transfer Requirements

While the content of this communication has been largely concerned with consideration of heat transfer, the data in FIGURE 10 emphasize the marked influence of additive type on the recovery of frozen and thawed cells. In each experimental series the weight of additive used is similar, that is, 11.8 per cent in the lactose and 12.5 per cent in the lactose-glucose preparations. The results indicate that glucose in combination with lactose provides protection to the cell for longer periods of time in the freeze-thaw cycle than does lactose alone. Nevertheless, lactose permits comparable red cell recoveries if the heat transfer requirements are met.

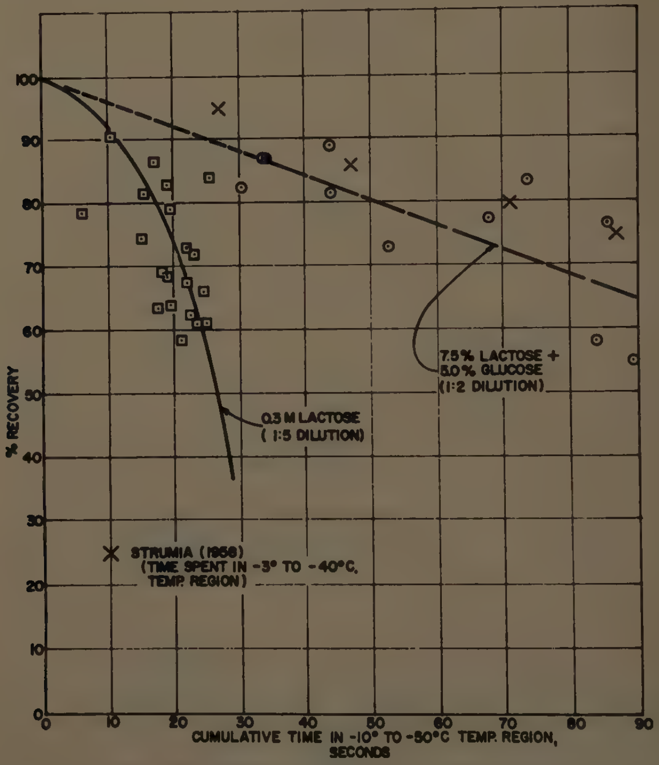

FIGURE 10. Plot of red cell recovery against the total time of residence (that is, during cooling and warming) of the central regions of volumes of blood within the temperature range 0° to −50° C. For further explanation of data of Strumia, see text.

The experiments with lactose as the sole protective additive during a freeze-thaw procedure are of further interest. This sugar does not penetrate the cell membrane. Consequently, its protective action is exerted in the extracellular environment. This in turn suggests that the events occurring within the cell in the course of freezing and thawing are not necessarily destructive to most of the erythrocytes, insofar as this is determinable by the extent of hemolysis at the conclusion of the process.

The increase in osmolarity following the addition of lactose to blood results in the movement of water from the cell, with a resulting concentration of the intracellular components. If intracellular ice formation accompanies the cooling process, a further concentration must occur. Since the substances present within the cell would include cations, it follows that their increased concentration does not lead inevitably to changes in the membrane permitting the release of hemoglobin. The time factor, if the situation described is valid, could be of critical importance, however, since the interaction between cations and membrane components should be time-dependent.

The considerably reduced time requirements for blood preparations containing glucose and lactose relative to those containing only lactose is also a point of interest. The permeability of the cell membrane to glucose ensures its presence within the cell during freezing and thawing. The time-extended protective action of glucose in combination with lactose suggests that intracellular events, while not necessarily important, can be critical if the necessary rates of heat transfer are not attained.

Discussion

In presenting data on the problem of heat transfer in blood it is the purpose of this communication to emphasize the quantitative aspects of the subject. There are, of course, many other facets involved in obtaining an erythrocyte with its complement of hemoglobin intact after freezing and thawing. Unless, however, those parts of the problem containing parameters that can be shown to be related to time-temperature are discussed in quantitative terms, considerable confusion can result.

On the basis of the results presented here it is concluded that the cells surviving a given rapid freeze-thaw process experience a relatively broad range of thermal histories. These varying histories suggest a heterogeneity of conditions throughout the specimen arising from the phase change during the cooling process. These could include differing rates of salt concentration, with the implication of hyper- or hypotonicity in different microregions of the freezing blood mass, regional differences in *p*H brought about by the crystallization, or precipitation of buffering salts, inequalities in pressures developed within the specimen as the phase transition occurs, and other consequences of the withdrawal of water as a solvent.

Although the red cell population contains many variables, their distribution through the liquid blood mass is probably uniform. Hence, all cell varieties experience the gamut of rates of temperature change in a freeze-thaw operation. The intact cells recovered from such a procedure are a population resistant to the particular stresses imposed. Whether this mode of population selection can be related positively to subsequent *in vivo* stability of the cells remains to be ascertained. In this regard, however, the reported work of Strumia[2] in humans and rabbits indicates that such is the case.

The rates at which heat is transferred to and from a cooling blood specimen are a critical factor affecting the erythrocyte in the processes that are commonly referred to as rapid freeze-thaw procedures. Under otherwise constant conditions, relatively small variations in geometry significantly affect these rates and therefore affect the red cell favorably or adversely, as the case may be.

Protective additives contribute to this phase of the problem by broadening the range of heat transfer rates to which blood may be subjected without increased hemolysis. Although some of the data presented may be regarded as supporting the concept of erythrocyte damage by salt concentration during the freezing process, other evidence requires caution in interpretation. Bricka and Bessis,[8] for example, have shown that polyvinylpyrrolidone, a polymer that does not penetrate the red cell membrane, permits the slow freezing and thawing of blood with less than 5 per cent hemolysis. Our own experience with this additive and others, including glycerol, the results of which will be reported elsewhere, suggests that the concept of cell destruction by salt concentration during freezing requires further examination. There can be no question that excessive concentrations of salt are capable of inducing eventual hemolysis. However, the possibility that this condition may be operative depends not only on the additive employed but on the rates at which heat is transferred to and from the specimen.

The maximum cross section that can be used in recovering a high percentage of cells from freeze-thaw processing, as determined by post-thaw hemolysis, has continued to increase. At one time it appeared that 1 to 2 mm., in droplet or other form, was an upper limit. As the use of thicknesses up to 17 mm. in work reported here will indicate, these small cross sections are no longer considered to be limiting.

Acknowledgments

I am indebted to many of my colleagues, in particular C. W. Cowley and G. F. Doebbler, for the experimental material discussed in this communication. My thanks are also due to L. D. Potts, who analyzed and programmed the droplet-freezing problem for solution on the Datatron computer.

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THE PHYSICAL STATE TRANSITION IN THE FREEZING OF LIVING CELLS*

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In the cooling of living cells the event of greatest interest is the change of tissue water from a liquid to a solid state. With minor exceptions, most cells show few harmful effects from being chilled for a short period, even at a rapid rate, down to the temperature of this phase change. Likewise, once most if not all of the available water of a given cell has been transformed into the solid state, with all the injury this entails, further cooling does relatively little additional damage.

Although the freezing of water is by no means the only event occurring in the temperature range of its phase shift, most of the important changes are the direct result of its crystallization or are closely associated with it. Such histomechanical and histochemical alterations as dehydration, concentration of electrolytes, denaturation of proteins, volume change or mechanical distortions, and redistribution of dissolved gases can all be attributed directly to the extraction from tissue elements of their liquid water by freezing.

The observations reported here were made in a study of the effects of altering the events that occur in the temperature range of phase transition upon the survival of cells after being cooled and rewarmed.

Under normal conditions it is the heat content of water that determines whether its molecules shall continue in random movement as a liquid or aggregate into rigid crystals. Furthermore, the number of crystals, their size, shape, and rate of growth may all be determined by the manner in which heat is removed. For this reason the most important tool in the study of freezing is the variation or manipulation of the cooling process.

However, the behavior of water, with respect to its crystallization, also may be modified by altering the pressure exerted upon it. To be effective, the hydrostatic pressures required for this are near the limit that can be tolerated by living cells. As serious as this limitation might be for the usefulness of compression in any practical way, it seemed worthwhile to investigate its possibilities as another tool in studying the effects of freezing on living cells.

The use before freezing of an additive such as glycerol can greatly increase the yield of surviving cells in most tissues. Although this protective treatment probably acts, in part, directly upon the physical process of phase change in which we are interested, it also has other physical and chemical effects on the cells, some of which are still poorly understood. To avoid these other effects, no additives were used in these experiments.

Our earlier experiences,[1,2] in agreement with those of Pomerat and Lewis,[3] of Rey,[4] and of others clearly showed that we could hope for only extremely small numbers of surviving cells from mammalian or avian cells frozen without the use of protective agents. Therefore, our decision to study freezing without pro-

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tective pretreatment necessitated the discovery of a method for detecting and measuring very low survival proportions. Dissociated tissue cells in liquid suspension seemed most adaptable for this purpose. One milliliter of a suspension containing 60,000 to 100,000 cells could be placed, after freezing and thawing, into a special microscope stage chamber and examined with phase-contrast illumination so that all surviving cells could be located and counted without fixation or staining. Since even a single surviving cell could be detected in this way, differences between treated samples of less than 0.01 per cent survival could be recorded.

The appearance of 2 surviving cells is shown in a photograph made 8 hours after freezing (FIGURE 1). The contrast between these 2 living cells and several obviously dead ones is clearly seen. In unfrozen control samples all cells spread out on the glass surface of the chamber within 8 to 12 hours. The activity of spreading was considered certain evidence of the viability of a cell. Rounded cells that did not spread after 24 hours were considered either dead or fatally injured by the experimental treatment. This assumption was verified in chambers kept for longer periods in which the unspread cells ruptured and disintegrated.

The tissues used were (1) cells of a strain obtained from human conjunctiva and kept in the laboratory as a stock culture, and (2) freshly dissociated chick embryo skin or other organ. It was soon noted that the survival percentage was 2 to 4 times higher if the cells were frozen immediately after trypsinization. This effect may be associated with a consolidation of the cell surface resulting from the state of cytoplasmic contraction caused by the trypsin treatment, since it gradually disappeared as the cells later spread. Also remarkable was the strikingly greater resistance to freezing injury of the established strain of human cells as compared to that shown by the freshly dissociated chick tissues.

To determine the proportion of cells that could be expected to survive freezing by cooling alone, sample suspensions were chilled at different rates in 2 types of containers. One of these was a thin-walled glass test tube into which was placed 0.5 ml. of cell suspension. This was used in the 3 experiments with chick skin that are summarized by means of the superimposed families of cooling curves presented in FIGURE 2. The test tubes were immersed successively in 2 coolants, the first varying in temperature with the experiment and the second at −78° C. In the upper group of curves *A* the 5 samples differed in the length of time taken to reach −30° C. Here there was a decrease in survival when the first cooling step took either a longer or a shorter time than about 5 min. Therefore, a 5-min. first-step cooling period was used for a second group of samples whose curves are shown at *B*. In this test the temperature of the first-step coolant was varied. It will be seen that, for the conditions used here, a coolant at −15° C. gave better survival than did the colder ones. In the last experiment (C) −15° C. was again used for the first coolant, but in this case the time in this coolant was varied.

It should be noted that in all 3 experiments 1-step cooling by direct immersion in the −78° C. coolant was far more harmful than 2-step cooling, which prolonged the stay of the cells in the phase-transition or crystallization temperature range.

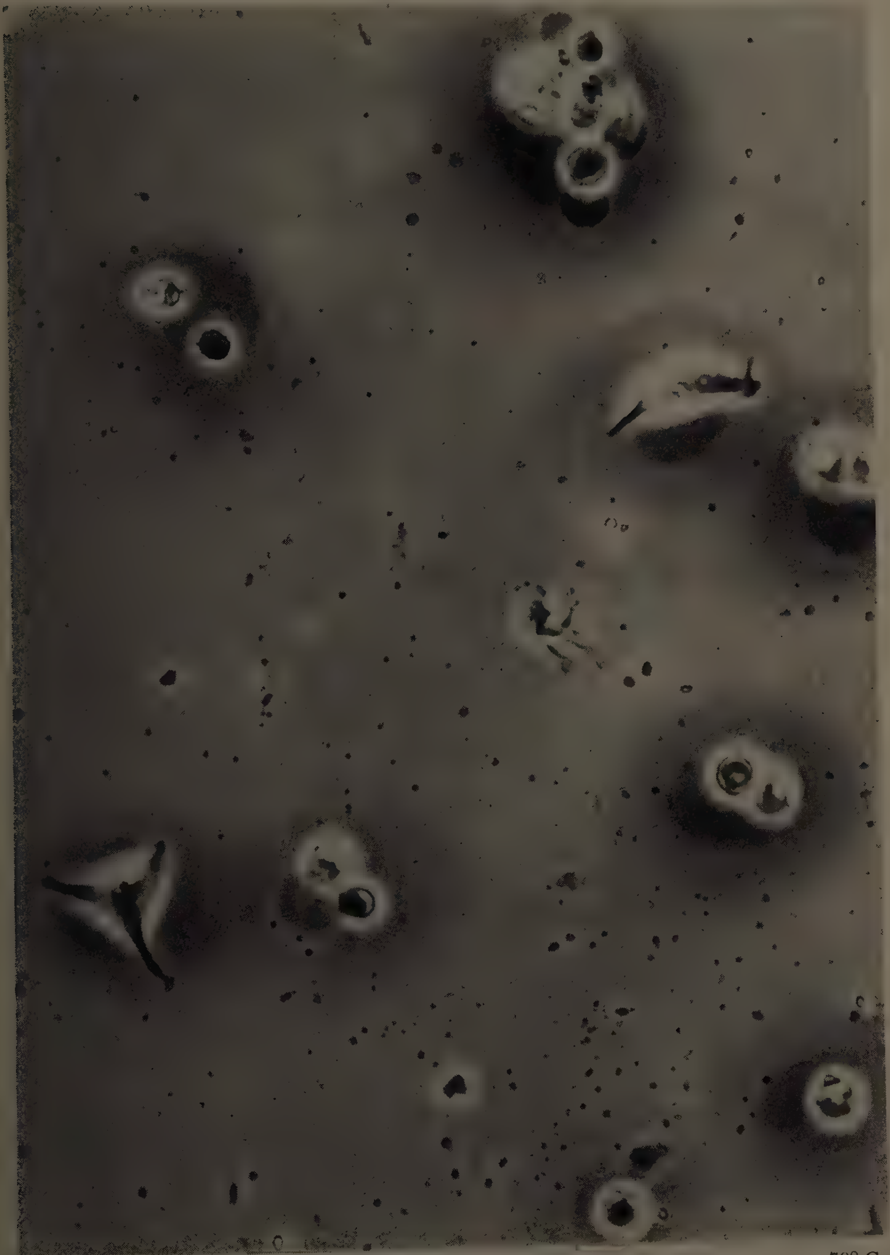

Figure 1. Human conjunctiva cells photographed 8 hours after being frozen at −78° C. Unstained. ×500.

The average percentage of survival in the 2 types of cells under the cooling conditions described is given in TABLE 1.

A second container used for cooling cells was a thin-walled polyethylene capsule 1.5 mm. in diameter and holding 0.02 ml. of suspension. The cooling curves of an experiment similar to the one above but using these capsules are shown in FIGURE 3. Because of the smaller volume of sample, the temperature drop here occurred more rapidly. The first curve in each family shows 1-step cooling by direct immersion in −78° C. coolant. The following 3 curves in each set show that cooling was detained, after freezing, at −15° or −5° C. for differing time intervals before the second-step temperature drop. The sur-

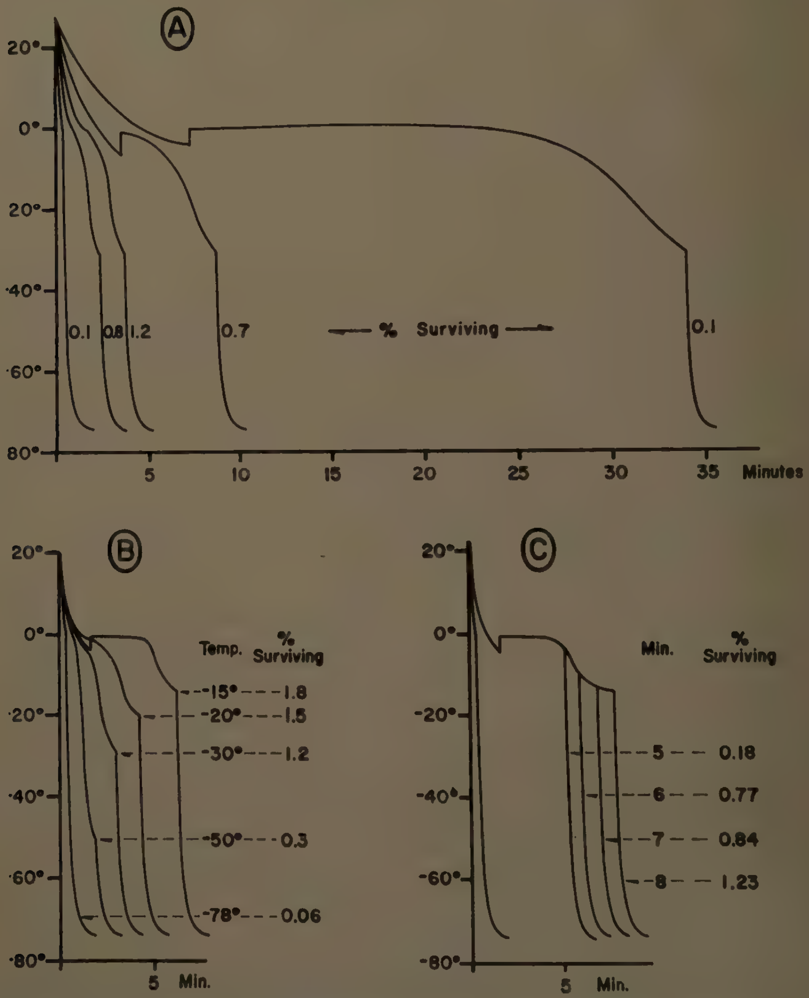

FIGURE 2. Cooling curves (in degrees centigrade) for chick skin cells contained in test tubes.

vival percentages are here much higher, since the cells used were human conjunctiva.

Three facts should be noted: (1) the poorest survival followed the most rapid or 1-step type cooling; (2) there is an optimal length of time for detaining the

TABLE 1

AVERAGE PERCENTAGE OF CELLS THAT SURVIVED FREEZING AND THAWING IN 38 EXPERIMENTS

Number of experiments	Cells tested	Average survival (%)	Range (%)
A* 2	Human conjunctiva	0.18	0.004–0.5
11	Chick skin	0.03	0.002–0.1
B† 15	Human conjunctiva	21.0	3.0–39.0
10	Chick skin	4.0	0.26–12.6

* Suspensions carried through the freezing temperature range in less than 2 sec.
† Suspension detained in freezing temperature range for 0.5 to 4 min.

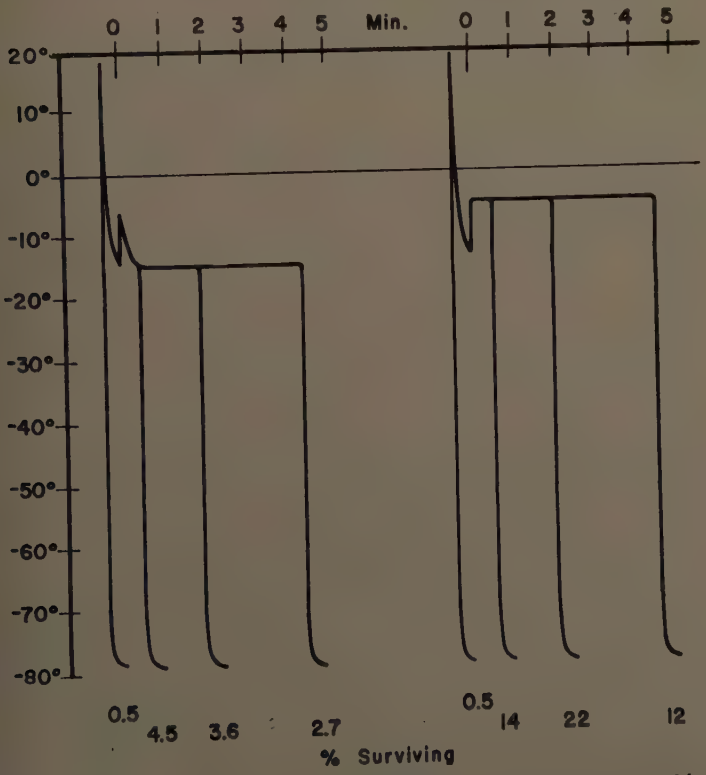

FIGURE 3. Curves obtained (in degrees centigrade) while cooling chick skin cells contained in plastic capsules. The first curve of each set shows the temperature drop on immersion directly in coolant at −78° C. In the remaining curves of each set cooling was detained at −15° and −5° C., respectively.

sample in the freezing temperature range; and (3) this optimal duration differs with the elevation of the detaining temperature.

These 3 facts were observed repeatedly in other experiments with both chick and human cells. The results in 2 such experiments are shown in the schematized diagram of FIGURE 4. Cell samples were cooled rapidly to the temperatures indicated by the broken lines. Each was then held at a constant temperature during an interval of time indicated by a dot or circle on the line. Each sample was then cooled quickly to −120° C. and then rewarmed; thus, the first broken line indicates that cell samples were cooled rapidly to −5° C. After maintaining the temperature of these for intervals of 1 sec., 30 sec., 2 min., and 5 min., respectively, each was cooled to −120° C. and then rewarmed. The survival percentage obtained for each sample treated in this way is indicated by the figure near the dot or circle standing for the time it was detained at that temperature level.

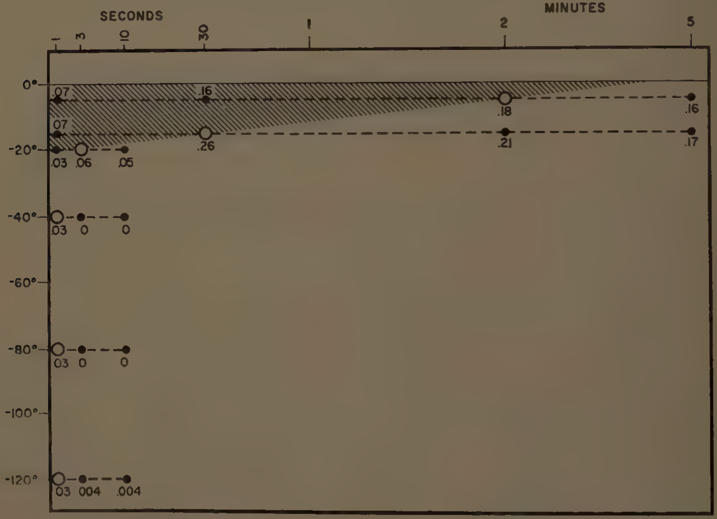

FIGURE 4. For explanation see text.

It will be seen that the optimal detention time for each temperature is represented by a circle. A line connecting the circles above −20° C. thus represents the time-temperature limit beyond which samples could not be carried without a resulting reduction in their survival. Therefore, the shaded area above this line covers a region on the chart in which cooling can be delayed with resulting improvement of survival.

The circles on the vertical line at the left of the chart are for cells cooled rapidly through the zone of crystallization and detained at temperatures below −20° C. Since these all suffered a reduction in survival by any detention, it was concluded that rapid cooling through temperatures below −20° C. was best.

It seems reasonable to assume from these facts that a certain minimal proportion of the water within or around a cell must be converted into ice before its temperature can be carried safely below the shaded area of this diagram. In the case of cells cooled through this "safe zone" at too rapid a rate, unfrozen water must then continue its phase transformation at a temperature that is somehow harmful.

This interpretation is also supported by the results from 2 experiments illustrated in FIGURE 5. The cooling curve here shown is that achieved by 1-step

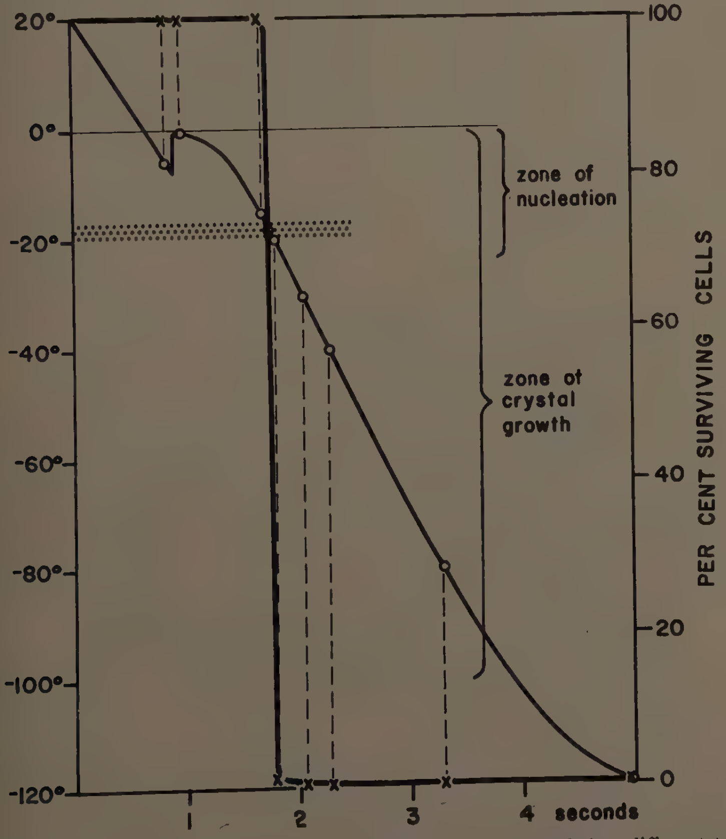

FIGURE 5. Diagram showing percentage survival in samples cooled to different temperatures centigrade. Circles on the cooling curve indicate temperature to which each sample was cooled. Corresponding crosses on survival curve show percentage of cells remaining alive in each sample after rewarming.

direct immersion which, as we have seen, results in very few surviving cells. A sample was cooled along this curve to the level indicated by one of the circles. At this temperature its cooling was stopped for 30 sec., after which it was rewarmed. The percentage of survival that was obtained from each of the samples thus cooled to a different temperature is indicated on the superimposed survival curve. The first 3 samples shown remained in the "safe zone" and, upon rewarming, exhibited no sign of injury despite the fact that 1 had reached a temperature of −15° C. and was not rewarmed until more than 30 sec. after freezing had commenced. The fourth sample was at −18° C., which is the border line of the safe zone indicated by a dotted band. Survival in it was approximately 75 per cent. All the remaining samples were carried below the safe zone, and they all showed survivals of less than 1 per cent. One cannot but notice the sharpness of the drop in the survival curve as cells were cooled through the dotted band, a fact that emphasized clearly that the major portion of freezing damage occurred in passing through a critical temperature zone near −20° C. It must, of course, be remembered that this was true only for the particular cooling rate shown. We have already seen that the situation was different for cells that had remained in the safe zone for a longer time. With higher-velocity cooling, the critical range might also be different.

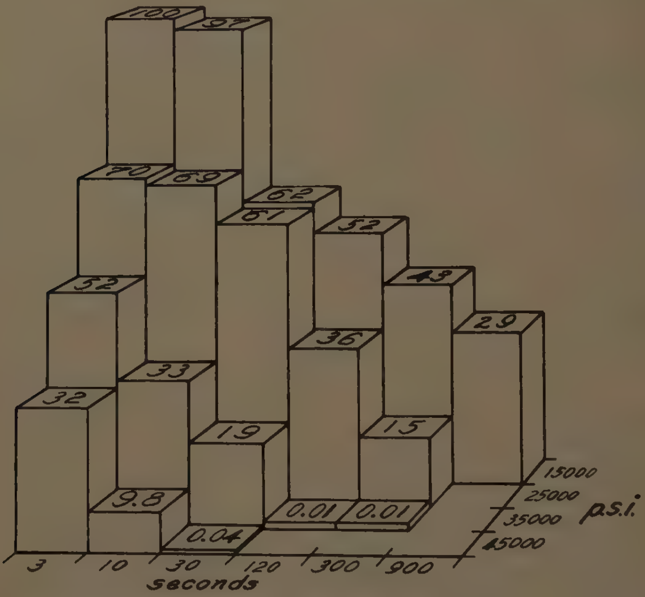

FIGURE 6. Percentage survival of chick skin cells after exposure to hydrostatic pressure.

This phenomenon raises the question of what may happen at more rapid cooling rates, perhaps 10 or 100 times as fast. These cooling velocities are difficult to achieve even with samples as small as 0.02 ml., and the temperature of the cooled sample cannot be arrested at a given point so readily.

For this reason it was hoped that, by the assistance of hydrostatic pressure, phase transition could be greatly accelerated within the zone of crystallization or the safe zone. This would permit us to test whether a subsequent temperature drop to lower levels could then be achieved without cell damage.

To accomplish this would require employing pressures as high as 30,000 to 35,000 psi, and it would first be necessary to determine the proportion of cells that might be expected to survive such an insult alone.

A group of such tests is summarized in the bar graph of FIGURE 6. From this it is quite apparent that such high pressures as would be needed could be used for only very short time intervals. After a pressure of 35,000 psi is applied for 3 sec., only about one half of the cells may be expected to live, while an exposure of 10 sec. reduces the proportion to one third. If we may assume that the injuries to cells by freezing and by high pressure are additive, then it would be possible to calculate the expected survival percentage when both stresses are applied to the same cells.

A phase diagram of water is presented in FIGURE 7. The phase boundary between ice I and water is seen to slope downward from 0° C. to the triple point of ice I, ice III, and water at −22° C. If a cell suspension were cooled to −20° C., it should be possible to thaw and refreeze the sample without further warming or cooling simply by increasing the hydrostatic pressure to 30,000 psi and then again releasing the pressure. This change would be independent of thermal conduction rates and would pass through the sample at the speed of an elastic deformation. When allowances were made for the heat equivalent of the mechanical work of compression and decompression, calculation showed that, of the 80 cal. of heat liberated by 1 gm. water in freezing at 0° C., only about 15 cal. would remain after carrying the water through the phase boundary between water and ice I at its lowest point and back to 0° C. It was hoped that, if the sample were simultaneously cooled, dissipation of heat by conduction would make possible almost instantaneous freezing and thawing.

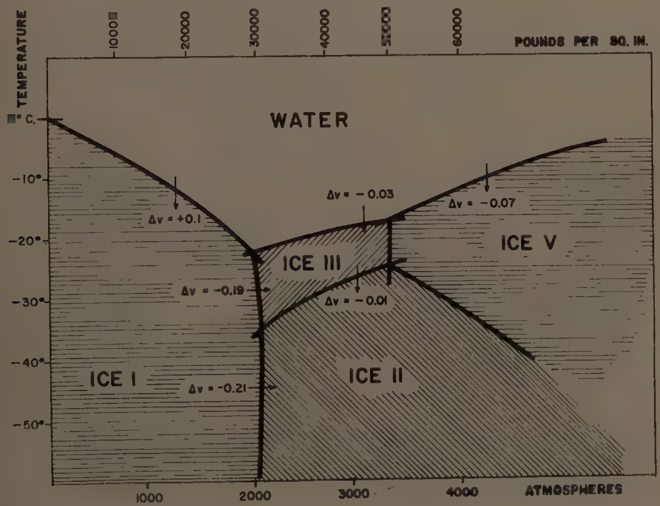

FIGURE 7. Phase diagram of water.

Oscilloscope tracings of the temperature changes in the same sample when it is first cooled without and then with added pressure make it possible to compare the rates of phase change under the 2 conditions (FIGURE 8). As judged by the convexity of the curve in *A*, crystallization was progressing during a 2-sec. time interval. In *B* 35,000 psi pressure was applied just above 0° C., with the observed resulting transitory temperature rise. Because of the depression of the freezing point of water under this pressure the sample remained liquid, while its temperature then fell to −22° C., at which point the pressure was released. The sudden temperature rise that then occurred is due to the heat of crystallization liberated by the sample. The facts that the temperature failed to rise to the melting point of physiological fluid and that the rise was immediately followed by a sharp temperature drop indicate that freezing had at least approached very close to completion. If so, there is compressed into less than 0.1 sec. both the "invasion" and "occupation" stages of freezing described by Luyet.[5]

A tracing made during the decompression freezing of human conjunctiva cells is shown in FIGURE 9. Since the survival obtained from this sample was the same as that expected if freezing and compression injuries are added together, no advantage of the rapid phase change is evidenced. A summary of the results of 16 experiments of this kind is shown in TABLE 2. The high observed value of 0.26 per cent in one group of experiments is attributable to a single case without which the average would be no greater than the expected percentage. There is no apparent explanation for this case.

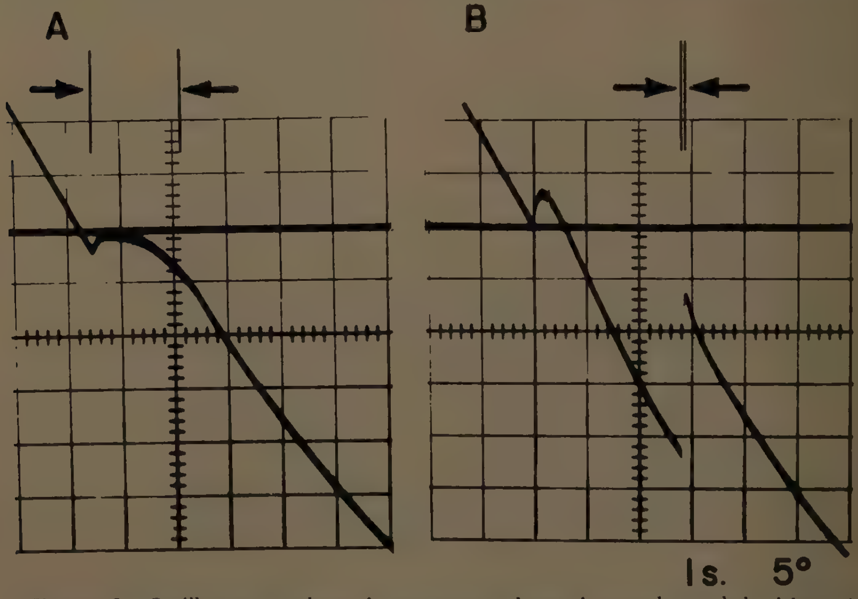

FIGURE 8. Oscilloscope tracings of temperature change in samples cooled without (*A*) and with (*B*) hydrostatic pressure. *Key:* dark horizontal trace, 0° C.; temperature interval, 5° C.; time interval, 1 sec. Phase transition time is indicated between arrows.

The failure of this acceleration of the freezing process to reduce the damage to cells may be interpreted in one of two ways: either the amount of ice formed by this procedure did not attain the minimum necessary to pass safely into regions of lower temperature, or the high velocity of the freezing is the damaging factor per se. If the latter explanation is correct, it would be in agreement with the observation frequently made for other tissue cells[6,7] that slow freezing gives better survival than does rapid freezing. It extends these observations by showing that even much more rapid freezing than heretofore attainable at a temperature above −20° C. does not yield better survival.

In view of this probability, the shaded area of FIGURE 4 may be safe by virtue of the reduced rate at which crystallization may take place at those temperatures. The protection afforded to tissues detained in this zone could thus be attributed to a reduction of the unfrozen water remaining available for more rapid crystallization at lower temperatures.

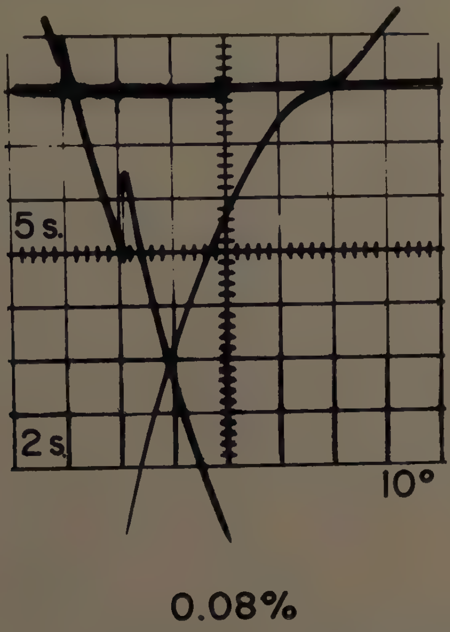

FIGURE 9. Pressure-assisted freezing curve with thawing curve of chick skin cells. *Key:* dark horizontal trace, 0° C.; temperature interval, 10° C.; time interval for freezing, 5 sec., for thawing, 2 sec.; indicated percentage, survival obtained.

TABLE 2

PERCENTAGE OF CELLS SURVIVING IN SUSPENSIONS FROZEN WITH DECOMPRESSION (35,000 PSI)

Number of tests	Tissues used	Average % surviving	
		Observed	Expected
5	Chick skin	0.00	0.01
5	Human conjunctiva	0.26	0.08
6	Human conjunctiva	0.08	0.08

If this be the correct line of reasoning, then there is no rationale for attempting to freeze cells at velocities greater than that occurring in the 2-step cooling process. The possible advantage of high or ultrahigh cooling velocities would

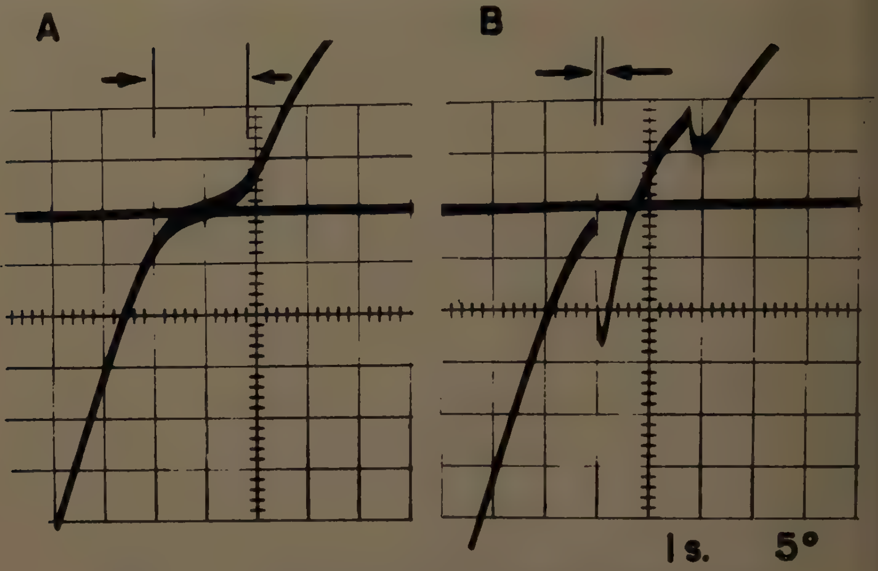

FIGURE 10. (*A*) Thawing with and (*B*) without hydrostatic pressure.

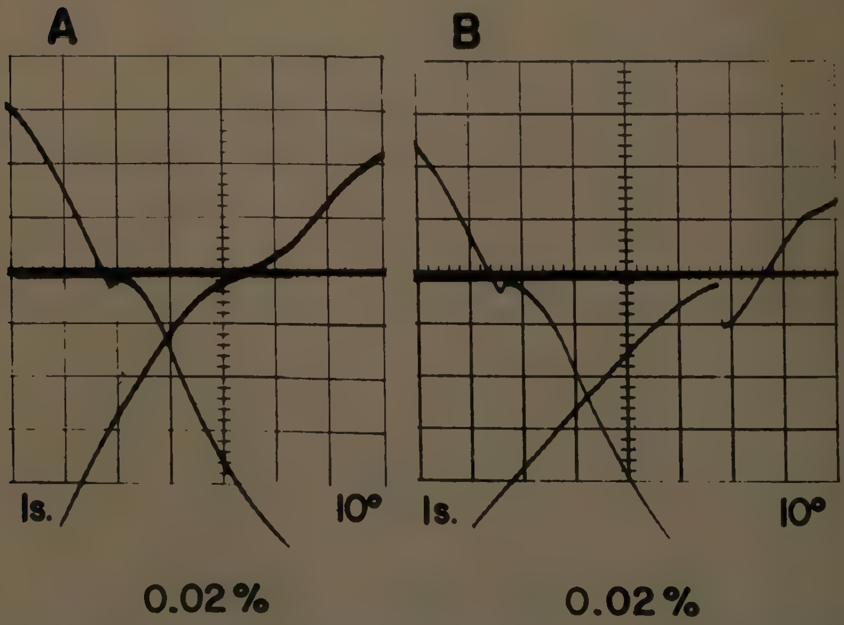

FIGURE 11. Freezing and thawing curves of chick skin cells. *Key: A*, without pressure; *B*, thawing with 35,000 psi hydrostatic pressure. The indicated percentages are the survivals obtained.

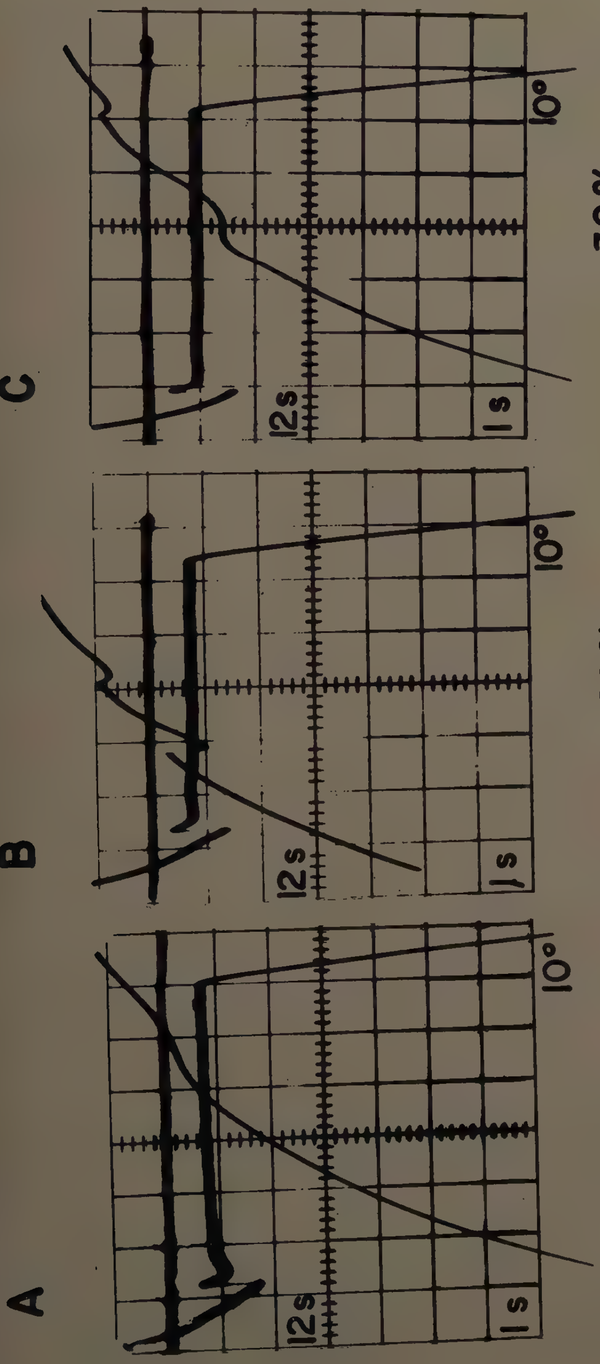

FIGURE 12. Freezing and thawing curves of human conjunctiva cells. *Key:* dark horizontal trace, 0° C.; temperature interval, 10° C.; freezing time interval, 12 sec.; thawing time interval, 1 sec. Indicated percentages are the survivals obtained.

lie, not in rapid crystallization of water, but in the possibility of bypassing the range of crystallization to enter that of vitrification.

While freezing with the aid of hydrostatic pressure may thus be theoretically the wrong thing to do, pressure-assisted thawing may still be beneficial. If, with pressure, thawing could be hastened, its use would be in line with the frequently demonstrated advantage of thawing at the highest speed attainable and might help to overcome the great difficulty encountered in adding heat to a frozen mass.

What 35,000 psi, when applied at the correct moment, can do to the thawing curve is shown in FIGURE 10. In *B* the pressure was applied to the sample when its temperature had risen to −5° C. and when, to judge by the character of its preceding warming curve, the sample was still largely in the solid state. The sudden temperature drop induced by the pressure is an expression of the uptake of heat by the sample in its change of state and, if our interpretation is correct, the drop shows that the sample had thawed completely in the time interval between the 2 vertical lines. The temperature of the thawed sample then rose rapidly, passing 0° C. without any sign of the plateau usually seen at this temperature. The temperature dip at +8° C. is due to the release of pressure.

TABLE 3

PERCENTAGE OF CELLS SURVIVING IN SUSPENSIONS THAWED WITH COMPRESSION (35,000 PSI)

Number of tests	Tissues used	Average % surviving	
		Observed	Expected
	Frozen rapidly		
8	Chick skin	0.03	0.01
16	Chick skin	0.026	0.03
16	Chick skin	0.005	0.02
	Freezing prolonged		
8	Human conjunctiva	33.0	18.0
8	Human conjunctiva	1.9	2.2

FIGURE 11 *B* shows the temperature tracing of chick skin cells thawed in this manner; a tracing of the control thawing is given at *A*. The indicated survival percentages show no evidence of benefit from the rapid thaw, but also no evidence of any injury by the compression. It is possible that the two effects balance each other in this experiment.

Pressure-assisted thawing was also combined with 2-step freezing, as shown in FIGURE 12. These are human conjunctiva cells detained in their cooling at −8° C. for about 1 min. In *A* is shown normal rapid thawing by immersion in water at +40° C. In *B* 35,000 psi pressure was applied at −4° C. which induced ultrarapid thawing. In *C* the same pressure was applied at −20° C.; thawing took place near the triple point with ice III (FIGURE 7). Here also, in all 3 cases the survival percentage is essentially the same. A summary of the thawing tests is given in TABLE 3. In 2 experiments there appears to be a decided advantage on the side of pressure-assisted thawing. In 2 cases the expected and observed results are nearly the same while, in another, pressure was harmful. Although not conclusive, these experiments suggest a possible reduction of cell damage in thawing by the accelerating effect of pressure. This may be true, however, only for the size of sample and container with which these tests were made.

Summary

Cell suspensions cooled to a temperature of −78° or −120° C. yielded the highest percentage of surviving cells when cooling was detained at temperatures above −20° C. for a length of time that varied with the detaining temperature.

Very few cells could survive rapid freezing by 1-step cooling.

Most of the injury from freezing occurred in a relatively narrow temperature zone which, for rapid freezing, is near −20° C.

Attempts to speed the formation of ice by the use of hydrostatic pressure were successful, but this failed to increase survival in the frozen sample. This observation is offered in further evidence that rapid crystallization is harmful to cells.

The assistance of pressure in hastening thawing may be of some advantage in preserving viability of cells.

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PHYSICAL FACTORS IMPLICATED IN THE DEATH OF MICRO-ORGANISMS AT SUBZERO TEMPERATURES*

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The effects of subzero temperatures on living cells and organisms are being studied with such increasing frequency in biological research as to justify the existence of a specialty of low-temperature biology. However, unlike the analogous field of low-temperature physics, low-temperature biology currently must be considered more a term of convenience than a description of a well-defined discipline. The physicist uses low temperature primarily as a powerful experimental tool to study the fundamental properties of matter, whereas the biologist's interest has been more pragmatic, and the reasons for his interest have been more varied. Cytologists and electron microscopists, on the one hand, use freezing and freeze-drying as methods of fixation to supplement the older procedures such as the use of osmium tetroxide.[1-3] Ecologists, on the other hand, are interested in learning how organisms or species in nature survive freezing temperatures.[4,5] Agronomists study the nature of frost damage in plants with the hope of developing frost-resistant varieties.[6] Food technologists are concerned with the effects of freezing, not only on food itself, but also on the bacterial population occurring in it.[7,8] Finally, many biologists use low-temperature storage and freeze-drying in an attempt to maintain a wide variety of cells and tissues viable and unchanged over long periods.[9,10]

However, regardless of whether a living cell is subjected to subzero temperatures by nature or by man, regardless of man's motives in doing so, and regardless of the type of cell, the end result is the same in that the cell finds itself exposed to a variety of physical and chemical phenomena that were not present beforehand. The nature of these phenomena and the effect they exert on the viability, structure, and function of the cell are subjects that properly belong to a field of low-temperature biology, and it is these subjects that I should like to discuss.

Physical Events at Low Temperatures

What physical and chemical events occur when an aqueous suspension of cells is cooled below 0° C.? First, almost by definition, a decrease in temperature decreases the kinetic energy of all the molecules in the system. The decrease in kinetic energy in turn results in secondary effects, including a reduction in the vapor pressure and density of water, an increase in the viscosity of water, and a decrease in the rates of chemical reactions. The magnitude of these changes is directly and continuously proportional to the degree to which the temperature is lowered.

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If the temperature is lowered sufficiently, a second major physical factor is almost always introduced; namely, the conversion of water into ice. Although this phase change cannot occur unless the temperature drops sufficiently low, the immediate physical consequences of ice formation occur independently of temperature and do so almost instantaneously; heat is released, the vapor pressure of the ice immediately drops below that of any supercooled water at the same temperature, its density abruptly falls, its specific heat is halved, and its viscosity rises one trillionfold. All of these effects and still others reflect changes in the molecular organization of water.

Temperature is, of course, one important factor in the freezing of water, since ordinarily water cannot freeze at a temperature above 0° C.* However, since water does not necessarily freeze even below 0° C., other factors must be involved. These others include the volume of water, the nature and number of solute molecules, the nature and number of nucleation centers, and the rate of cooling. Similar factors influence the size, number, and habit of the resulting ice crystals.

A description of the physical events becomes more complex for systems containing living cells, for then one must consider that low temperatures act, not only on the water and solute molecules outside the cell, but also on those within it, the two areas being separated by a membrane exhibiting highly specific permeability to the various molecular species present. Moreover, the solute molecules within the cell differ in number, type, and organization from those on the outside, and even the structure and organization of water may be different in the two regions.[13,14]

Sooner or later the cooled cells will probably be returned to normal temperatures to determine how they have been affected, and this warming introduces a whole new set of physical events: the kinetic energy of the molecules increases, and any ice melts to water.

Physical Factors Implicated in Death

These then, are the major physical phenomena to which a cell is likely to be exposed when cooled to below 0° C. The question naturally arises as to which of them affect structure and function and thus determine whether or not the cell will survive. Experiments must provide the answer. Accordingly, most of the remainder of this discussion is concerned with presenting and interpreting the data from certain experiments in an attempt to arrive at a plausible answer. The experiments will concern chiefly the cells of three species of microorganisms, and the answers, therefore, may well be applicable to cells of only these three species: the yeast *Saccharomyces cerevisiae*, the bacterium *Pasteurella tularensis*, and spores of the fungus *Aspergillus flavus*.[15,16]

One advantage of using microorganisms is the comparative ease with which survival can be determined. A second advantage is that many of them can be successfully suspended in distilled water. The use of pure water as a suspending medium reduces the number of physicochemical factors interacting at subzero temperatures and, in so doing, simplifies the interpretation of the results.

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* However, under certain conditions icelike compounds (clathrates) can exist at temperatures well above 0° C.[11,12]

Double-distilled water was the suspending medium for the yeast cells and *Aspergillus* spores, except when otherwise noted. The *Pasteurella* cells were suspended in a gelatin-saline solution because a pure water medium was harmful to them.

One cannot make a simple statement about the susceptibility or resistance of these cells to subzero temperature exposure because the percentage of survival ranges from almost zero to nearly 100 per cent, depending on experimental variables to be discussed shortly. What we should like to know is which of the several physical consequences of exposure to low temperatures are responsible for death when it does occur. I have mentioned that these consequences fall into two major categories: those derived from changes in the kinetic energy of molecules or temperature itself, and those dependent on phase changes, specifically the freezing of water and the melting of ice. Therefore, the first question is whether low-temperature death is a direct consequence of freezing and melting. The question itself suggests the experiment needed to provide the answer; namely, a comparison of the percentage of cells that survive low temperatures in the absence of ice formation with the percentage that survive in its presence. The comparison is made for *Aspergillus* spores and yeast cells in TABLE 1. The most significant aspect of these data is that survival was always close to 100 per cent in all cases when the external medium remained unfrozen irrespective of the temperature to which the cells had been cooled. On the other hand, survival was not always high when the external medium was frozen but ranged widely from less than 1 per cent to nearly 100 per cent. The significance of this great range will be discussed further. For the moment, the important

TABLE 1

SURVIVAL OF SPORES OF *ASPERGILLUS FLAVUS* AND CELLS OF *SACCHAROMYCES CEREVISIAE* AFTER BEING RAPIDLY COOLED AND SLOWLY WARMED IN EITHER FROZEN OR UNFROZEN SUSPENDING MEDIA

Suspending medium	Temperature (°C.)	Organism	Percentage survival* in:	
			Unfrozen medium	Frozen medium
Water	−5	*A. flavus*	100	102
		Yeast	105	98
	−10	*A. flavus*	100	100
		Yeast	102	85
	−15	Yeast	99	23–60
	−20	*A. flavus*	—	67
		Yeast	—	10
	−40	*A. flavus*	—	11
		Yeast		<1
4.0 molal $CaCl_2$	−45	*A. flavus*	86	—
	−60		—	17.4
3.3 molal $MgCl_2$	−26	*A. flavus*	83	—
	−38		—	8.6

* Based on a value of 100 per cent for the survival of cells in unfrozen water at room temperature.

conclusion is that little death occurred in the absence of freezing; hence, low temperatures and the accompanying physicochemical consequences of decreased molecular kinetic energy appear to be harmless in themselves. Alternatively, one could say that the freezing of water is, in the logicians's sense, necessary but not sufficient for death.

The suggestion that death is associated with freezing immediately raises two further considerations. In the first place, since freezing can be extracellular or intracellular, the location of the ice could be an important factor. In the second place, freezing is, in a sense, a two-sided process. On the one hand, it yields increasing amounts of ice with physical properties and characteristics quite different from those of water. Cell injury might be directly related to these altered properties of the solid state, as for example, the increased volume of ice. On the other hand, the process of freezing also results in the progressive disappearance of water, with the result that any solutes in the water become progressively more concentrated. Thus, cell injury might be associated with this removal of liquid water and the concomitant rise in solute concentration rather than with the ice formation responsible for the water loss.

To decide among these possibilities it will first be necessary to examine experimental data on how survival is related to three experimental variables; namely, the minimum temperature to which cells are cooled, the rate of cooling, and the duration of exposure. The relationship between survival and the first of these, the minimum temperature, is summarized in FIGURE 1. The relationship was much the same in all these microorganisms. Survivals remained relatively high down to a certain critical zone of temperatures, whereupon they dropped sharply and suddenly. The chief differences among the 3 cells were the specific temperatures that delimited the critical zone. The survival of yeast cells remained nearly 100 per cent down to −10° C. and then dropped sharply between −10° and −25° C. The drop in survival of *Aspergillus* spores occurred between −10° and −40° C.[15] and that of *Pasteurella* cells between −30° and −45° C.[16] The fact that the drop in survival occurred within a rather narrow range of temperatures and not gradually over the whole range studied gives further support to the conclusion that death is associated with a phase change.

It should be noted that the temperature zone in which survival decreased sharply did not coincide with the temperature at which extracellular freezing occurred, for the external medium was frozen at every temperature used, with freezing either having been spontaneous or else initiated by seeding.

The lethal consequences of cooling to temperatures below the critical zone occurred extremely rapidly. In yeast, for example (FIGURE 2), almost all the harmful effects of rapid cooling to −30° C. occurrred during the first minute of exposure, with survival dropping from 100 to 1 per cent in that short interval. There was little further decline even when the cells were held an additional 90 min. at −30° C.

The rate of cooling in all of these experiments was rapid (50° C./min. or higher to −30° C. or below). If, instead, the cells were cooled more slowly at, for example, 1°/min., the damage at temperatures below the critical zone was not nearly so extensive (TABLE 2). With *Aspergillus* spores, for example, rapid cooling to −70° C. yielded 7 per cent survival, whereas slow cooling yielded 34 per cent.[15] The survival of rapidly cooled *Pasteurella* cells was only one three-thousandth that of slowly cooled cells,[16] and the survival of rapidly cooled yeast cells was only one ten-thousandth that of slowly cooled cells.

In summary, then, death did not occur in the absence of extracellular freezing but, even in the presence of external freezing, extensive injury occurred only if the temperature dropped below a critical zone. The drop in survival occurred abruptly with respect to both temperature and time. Finally, when cooling was slow, the drop in survival was much less extensive than when cooling was rapid.

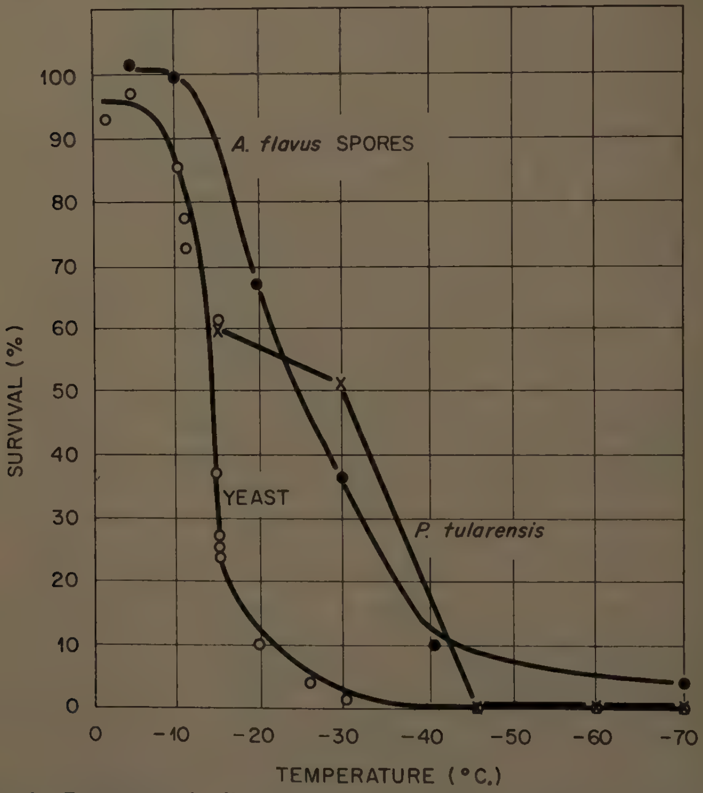

Figure 1. Percentage of cells surviving rapid cooling to indicated temperatures and subsequent slow warming.

These observations may provide answers to the two questions raised earlier. One question is whether death is associated with extracellular or intracellular freezing. The other is whether death is a direct consequence of ice formation or is a result of exposure to the high concentration of solutes produced secondarily by freezing.

The evidence, I believe, strongly suggests that death was not the result of high concentrations of solutes. If it were, one would expect longer exposures to produce greater damage, but the reverse actually occurred. Slow cooling was less harmful than rapid cooling, even though slow cooling resulted in the longer exposure. Moreover, even with rapid cooling the damage occurred almost entirely within the first minutes of exposure and did not increase significantly with time.

In the case of *Aspergillus* spores there is even more positive evidence against the supposition that a high concentration of solutes is the lethal factor. As shown in TABLE 3, these spores were quite capable of surviving contact with

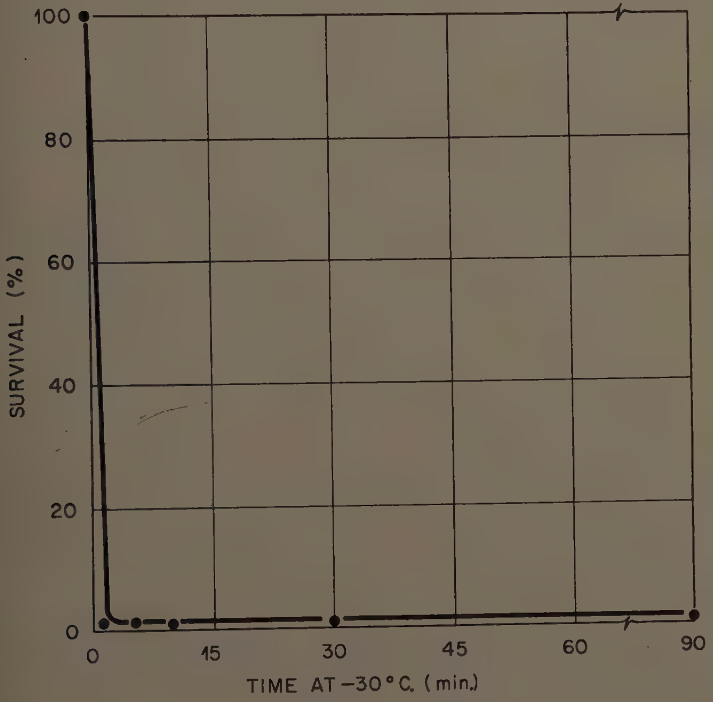

FIGURE 2. Survival of cells of *Saccharomyces cerevisiae* as a function of duration of exposure at −30° C. Both cooling and warming were rapid.

TABLE 2

EFFECT OF COOLING RATE ON THE SURVIVAL OF CELLS COOLED TO −75° C. AND WARMED SLOWLY

Organism	Percentage survival with:	
	Slow cooling (1° C./min.)	Rapid cooling (300° C./min.)
Aspergillus flavus (spores)	34.3*	7.1
Pasteurella tularensis	2.0	0.0006
Saccharomyces cerevisiae	0.93	0.0001

* The cooling rate was 0.4° C./min. to −46° C. and about 20° C./min. from −46° to −65° C.

concentrated solutions of $CaCl_2$ and $MgCl_2$ at low temperatures provided the solutions remained unfrozen.[15]

The other question is whether cell injury is a consequence of extracellular or intracellular freezing. The finding that low temperatures were harmless in the absence of extracellular freezing would indicate that extracellular freezing was at least a prerequisite for damage. Nevertheless, it is difficult to see how it could have been directly associated with death simply because death was not an invariable consequence of external freezing. With yeast, for example (FIGURE 1) almost all of the cells survived cooling to −5° C., but only 4 per cent survived cooling to −25° C. even though the external medium was frozen in both cases. Moreover, since the medium was deionized distilled water, one would expect it to be frozen completely at both temperatures.

This last statement could be challenged on the grounds that the water surrounding the cells, in all probability, would not remain pure, but would become a dilute aqueous solution because of solutes eluted and extracted from the cells. If the external medium was in fact a solution, even though a dilute one, it would contain more ice at −25° C. than at −5° C., and the unfrozen portion would contain a higher concentration of solutes, a concentration perhaps sufficiently high to be harmful. This argument could well be valid except that we have already noted the difficulties in ascribing the death of these cells to exposure to high concentrations of solute.

TABLE 3

SURVIVAL OF *ASPERGILLUS FLAVUS* SPORES IN UNFROZEN AND FROZEN EUTECTIC SOLUTIONS OF $CaCl_2$ AND $MgCl_2$

Solution	Temperature (°C.)	Percentage survival with:	
		Medium unfrozen	Medium frozen
4.0 Molal calcium chloride	+25	83.7	—
	−45	80.5	—
	−60	—	14.6
3.3 Molal magnesium chloride	+25	78.3	—
	−26	80.1	—
	−38	—	6.7

Solutions are also characterized by having a eutectic point, the temperature below which no liquid solution can exist. It might be argued that the critical temperature zone in which survival drops so sharply represents the attainment of the eutectic point of the external medium. In other words, perhaps the death of these cells is a direct consequence, not of extracellular freezing per se, but of the completion of extracellular freezing at the eutectic point. The evidence on hand does not rule out such a hypothesis, but it does present two arguments against it. One argument is that the survival of the yeast cells and *Aspergillus* spores began to drop sharply at about −10° C., but −10° C. would almost certainly be considerably above the eutectic point of a solution containing those solutes likely to be removed from cells. The second argument is that survival was greatly dependent on the rate of cooling, but a eutectic point is fixed for a given solution, and should in no way be modified by the cooling rate.

If death is associated with the freezing of water, but not with extracellular freezing, it seems logical to hypothesize that it is a consequence of intracellular freezing. On the basis of such a hypothesis, the chronological events in low-temperature exposure would be pictured as follows:

As a suspension of cells in water or aqueous solutions is cooled below 0° C., both the extracellular and intracellular water at first remain unfrozen because of supercooling and, as long as they remain unfrozen, the low temperatures are not injurious. At some temperature the extracellular water freezes, the temperature depending on the nature of the medium and on whether it is purposely seeded or freezes spontaneously (with water as the suspending medium, spontaneous freezing occurred at −10° to −15° C.). The extracellular freezing then somehow initiates the freezing of the water inside the cell, provided that the temperature of the cells is sufficiently low and provided that the cooling rate has been sufficiently high. What we are suggesting is that the water within the cells has a strong tendency to supercool, possibly because there is little likelihood of a nucleation center existing in the tiny volume of water contained within any given cell. When extracellular ice appears, however, it acts as a nucleation center and seeds the intracellular water, causing it to freeze.

Two objections could be raised immediately to the idea of extracellular ice seeding intracellular water. One objection is that studies such as those by Luyet and Gibbs[17] and by Chambers and Hale[18] on living cells indicate that the cell membrane is an effective barrier against such seeding. Second, even if seeding could occur across the membranes of these cells, why would such seeding not occur whenever extracellular ice was present, irrespective of temperature; that is, why would it be necessary for the temperature to drop to the critical zone (below −10° C.) before seeding and intracellular freezing could occur?

Fortunately, there is a plausible answer to these formidable objections, and it may be stated as follows. Cell membranes are believed by many authorities to contain water-filled channels.[19] The diameters of these channels, whatever their exact nature, are certainly small—measured in angstrom units. As a result, if an external ice crystal is to "grow" through such a space to seed the water inside the cell, the tip of that crystal must assume an extremely small radius of curvature, a radius measured in angstrom units. The equilibrium freezing or melting point of a crystal of such radius would no longer be 0° C., but would be a much lower temperature—possibly as low as −30° C.[20] In general, for every radius of curvature there is a critical temperature at which ice and water are in equilibrium.* If a crystal of given radius is below the critical temperature, it will grow; if it is above the critical temperature, it will melt. Suppose that the radius of an aqueous channel in the cell membrane of these cells is 25 Å (Parpart and Ballentine[19] believe they are even narrower than this in red cells); then it would be necessary for the tip of an ice crystal to have a radius of curvature of less than 25 Å in order to pass through the channel, as depicted schematically in FIGURE 3. The critical temperature for a radius of

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* This is the reverse of the customary way of expressing the concept. The usual statement is that for every subzero temperature there is a critical radius of curvature at which ice and water are in equilibrium.[20]

25 Å is −15° C.; hence, above that temperature a tip of the required radius could not exist, and the membrane would be a barrier to seeding. Below −15° C. a crystal of the required radius not only could exist, but could grow through the aqueous channel in the membrane and initiate freezing within the cell. In more general terms a cell membrane could be a barrier to seeding at temperatures nearer 0° C. and cease to be a barrier at lower temperatures.* Model membranes of regenerated cellulose and gelatin have been found by Lusena and Cook to act as partial barriers to seeding in this sense.[21]

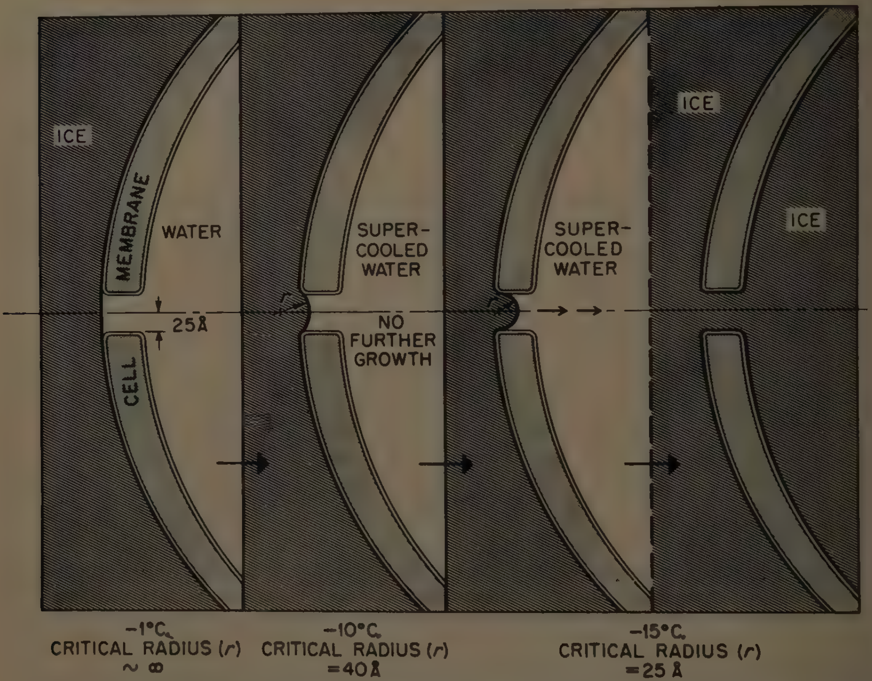

FIGURE 3. Schematic drawing of hypothetical growth of extracellular ice crystal through aqueous channel in cell membrane and subsequent seeding of intracellular water. Ice crystal of required radius of curvature can exist only at −15° C. or below.

A second item that must be accounted for by the hypothesis of intracellular freezing is the observed protective effect of slow cooling. The obvious supposition would be that slow cooling reduces the likelihood of intracellular freezing, and there is considerable evidence that it can, in fact, do so. Nei has published photographs showing intracellular freezing in rapidly cooled yeast cells, but not in slowly cooled ones.[22] In the latter case the cells were shrunken and flattened, presumably because of water loss. Meryman and Platt, among others, have described similar consequences of rapid and slow cooling in liver and other mammalian cells.[23]

Apparently what happens during cooling is the following sequence of events. When the water in the external medium freezes, its vapor pressure immediately drops below that of the supercooled water in the cells. The resulting vapor-pressure differential produces a tendency for water to flow out of the cell. If cooling is slow there is sufficient time for an appreciable quantity of water to leave the cells before they cool to the temperature at which intracellular freezing would ordinarily occur. By the time that temperature is reached, the residual intracellular solution is incapable of freezing, possibly because the resulting concentration of solutes has lowered the freezing point, or possibly because the mobility and activity of the water molecules have been reduced by other factors to such an extent that nucleation and crystal growth become improbable or impossible. On the other hand, with rapid cooling there is not time for sufficient water to leave the cell to prevent intracellular freezing from occurring when the temperature drops sufficiently.

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* In the case of the microorganisms the barrier could be plasma membrane or the cell wall.

The discussion thus far has been concerned with the physical factors that operate during cooling; however, in most cases the cells will sooner or later be warmed and subjected to the stresses involved in warming and the melting of ice. The warming phase of low-temperature treatment has received comparatively little attention from investigators; yet its effects can be not only important, but they can be the most important factors in survival in the entire low-temperature sequence. Most of the experiments presented thus far have subjected the cell suspensions to slow warming at about 1° C./min. Very different results were obtained when cells were warmed rapidly at rates of 500° C./min. or more; namely, a high percentage of cells survived irrespective of either the rate of cooling or the temperature to which they were cooled.

For example (FIGURE 4), when *Pasteurella* cells were warmed rapidly, a relatively high proportion survived regardless of whether initial cooling had been rapid (300° C./min.) or slow (1° C./min.).[16] When the cells were warmed slowly, not only was the percentage of survivors much lower, but it was more dependent on the cooling rate. Similarly (FIGURE 5), with rapid warming, recoveries were high regardless of the temperature to which the cells had been cooled, whereas with slow warming, temperature was a highly critical factor, as shown by the fact that survivals plummeted below −30° C.

The relationship between the rate of warming and the survival of *Pasteurella* cells is shown in FIGURE 6 in more detail. The degree of injury associated with the lowest warming rates depended greatly on the rate of cooling and the minimum temperature, as has been discussed earlier; however, whenever slow warming yielded low survivals, higher warming rates yielded progressively higher survivals. In fact, when the cells were warmed at the highest rates of about 1000° C./min., there was comparatively little damage.

The effect of warming rate on the viability of *Aspergillus* spores was similar (FIGURE 7).[24] Once again, when slow warming yielded low survivals, higher rates of warming yielded higher survivals. The survival of yeast cells was not as dramatically influenced by warming rate (TABLE 4), yet even in this case rapid warming was many times less harmful than slow warming.

The fact that extensive damage to *Aspergillus* and *Pasteurella* cells could occur only when warming was slow and never occurred when warming was rapid leads to the interesting conclusion that most of these cells survived the cooling phase of low-temperature exposure. This must be true because, if all killing had occurred during cooling, variations in the rate of subsequent warming could have had no effect on the percentage of cells surviving. Consequently, in the last analysis, whether death occurred depended to a large extent on factors operating during warming, factors that were influenced by the warming velocity.

Perhaps the clearest way to picture the deleterious action of low temperatures on these cells is to conceive of death as the end result of a sequential series of specific events, much as a biochemical product is the end result of a sequential series of enzyme-catalyzed reactions. If any one of these events is prevented from occurring, death becomes much less likely. The sequence of steps leading to the death of these microorganisms is (1) extracellular ice formation, (2) a sufficiently low temperature, (3) a sufficiently high rate of cooling, and (4) a sufficiently low rate of warming. The first three steps take place during cooling, and we have hypothesized that their end product is the formation of intracellular ice. However, since death (at least of *Aspergillus* spores and *Pasteurella* cells) also requires the fourth step of slow warming, we are forced to conclude that many of the cells are not killed outright by the formation of this internal ice. Whether they are killed depends somehow on events taking place during warming.

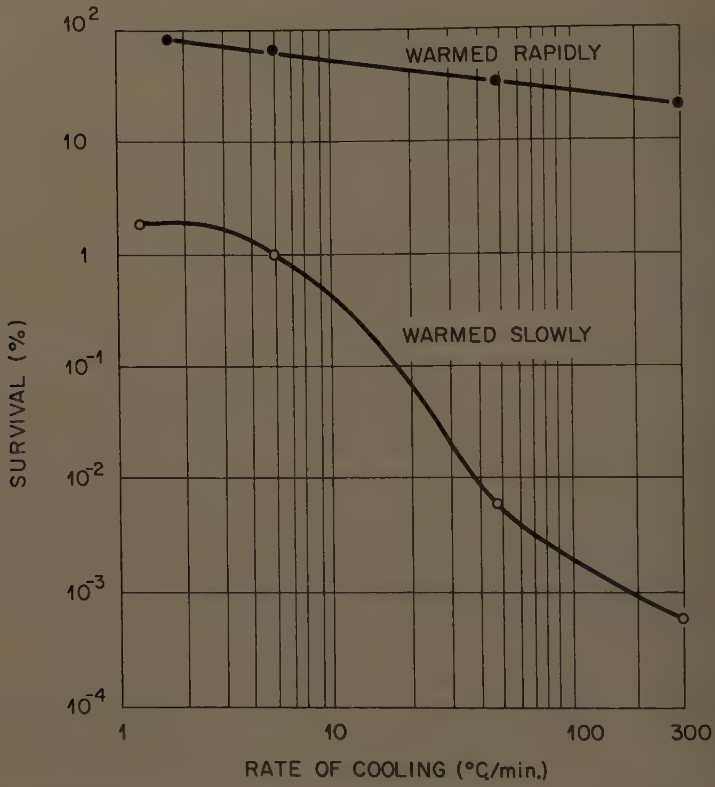

FIGURE 4. Survival of *Pasteurella tularensis* cells cooled at indicated rates to −75° C. and warmed either rapidly or slowly.

At present one can only speculate as to what sort of events these might be. One possibility is that the effects of rate of warming are related to the process known as migratory recrystallization.[25] This process is the growth of larger crystals at the expense of neighboring smaller crystals at subzero temperatures. We have hypothesized that these microorganisms supercool extensively before undergoing internal freezing and have suggested that nucleation occurs through the aqueous channels of the cell membrane. Since there are probably hundreds of thousands of these channels, nucleation would be likely to occur at many points in the cell, and the greater the number of nucleation centers, the smaller and more numerous would be the resulting ice crystals. Possibly the resulting crystals would be sufficiently small to be harmless to the cell. During subsequent warming, however, there might be time for the crystals to grow by migratory recrystallization to a damaging size before they melted if the warming were carried out slowly, but not if it were carried out rapidly.

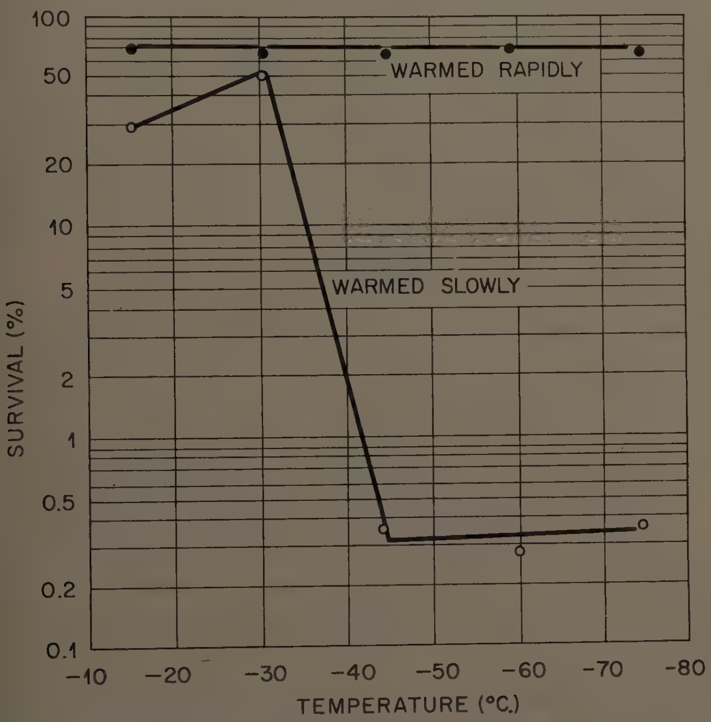

Figure 5. Survival of *Pasteurella tularensis* cells cooled slowly to indicated temperatures and warmed either rapidly or slowly.

Published descriptions of intracellular freezing by Chambers and Hale,[18] Luyet and Gibbs,[17] Smith and her co-workers,[26,27] and others all mention the fact that under the microscope this freezing is observed as a sudden "blacking out" of the cell. The blacking out means that most of the light that strikes the undersurface of the cells is being scattered or reflected and suggests that the cell contains numerous small ice crystals. Also interesting are the further observations by Chambers and Hale on amoebae and onion epidermis[18] and by Luyet and Gibbs[17] on onion epidermis that the opacity of the frozen cells soon diminishes as the small crystals grow into or join to form larger, more transparent ones. This process may or may not be true migratory recrystallization,

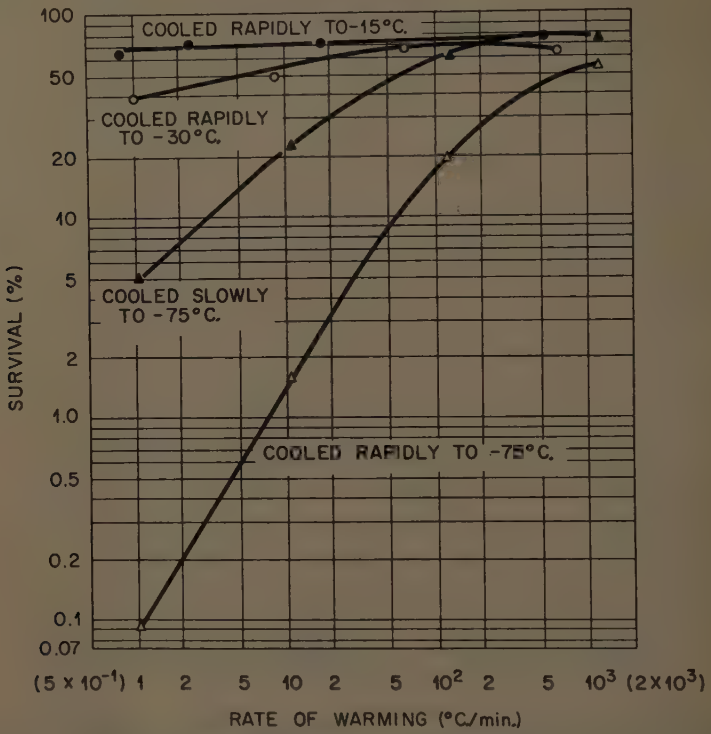

FIGURE 6. Survival of *Pasteurella tularensis* cells warmed at various rates from −15° −30°, or −75° C. Redrawn from Mazur *et al.*[16] by permission from Academic Press, Inc., New York, N. Y.

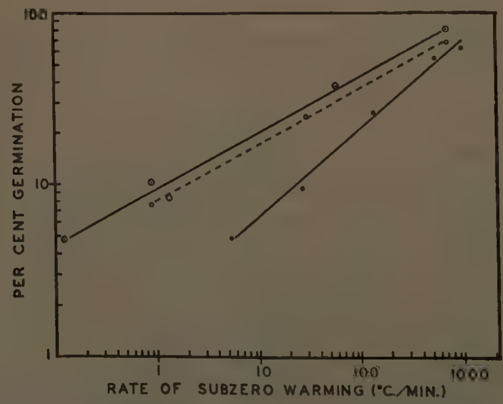

FIGURE 7. Percentage of *Aspergillus flavus* spores germinating after being warmed at indicated rates from −70° C. Initial cooling was rapid. Three experiments are shown. Reproduced from Mazur[24] by permission from the *Journal of General Physiology*.

but the observation is nevertheless significant in showing that ice crystal enlargement can occur in cells held at a given subzero temperature.

It must be emphasized that this speculation on migratory recrystallization is meant only to be an example of one process that could be influenced by the rate of warming and that conceivably could be the cause of the deleterious effect of slow warming on the 3 microorganisms. As yet we have no experimental data to present either for or against the idea.

Influence of the Suspending Medium

Most of the physical factors implicated in low-temperature death are dependent on the properties and behavior of water and ice. Consequently, it would not be surprising to find that substances that affect these properties or behavior may also affect survival. The now classic example, of course, is the protection resulting from the presence of glycerol in the suspending medium.

The survival of the 3 microorganisms under discussion after low-temperature exposure is greatly affected by the nature of the suspending medium. A good example is the response of *Pasteurella* cells when solutions of glucose or lactose were used as suspending media in place of the gelatin-saline solution used routinely (FIGURE 8).[28] The precipitous drop in survival between −30° and −45° C. disappeared when the external solution contained sugars, and survival was comparatively high at all temperatures. The spores of *Aspergillus* were similarly protected when suspended in 40 per cent ethylene glycol (TABLE 5).

TABLE 4

EFFECT OF RATE OF COOLING AND RATE OF WARMING ON THE SURVIVAL OF CELLS OF *SACCHAROMYCES CEREVISIAE* COOLED TO −75° C.

Rate of cooling	Rate of warming	Percentage survival
Rapid (280° C./min.)	Slow (1° C./min.)	0.0001
	Rapid (1100° C./min.)	0.22
Slow (1.4° C./min.)	Slow (1° C./min.)	0.93
	Rapid (1100° C./min.)	16.7

The protective actions of various solutes on various cells are observed facts, but the fundamental basis of protection generally remains obscure or unknown. Only in the case of glycerol is there a well-supported hypothesis, thanks mostly to the excellent work of Lovelock on red blood cells[29] and vertebrate spermatozoa.[30] According to this hypothesis, low temperatures are harmful because they produce high solute concentrations, and glycerol protects because it is itself innocuous and because its presence reduces the concentration of other solutes at any given subzero temperature.

If high solute concentrations were not the cause of death in the three microorganisms, as appears to be the case, the basis of the protective action of the sugars and of ethylene glycol could not have been that which Lovelock postulates for glycerol in animal cells.[30] Since we have hypothesized that intracellular freezing is a prerequisite for death, the simplest supposition would be that these two substances protect the cells by preventing or reducing the likelihood of intracellular freezing. One way that they could have done so would have been to prevent the postulated seeding through the cell membrane. Lusena and Cook, in their study of seeding across model membranes, found that ice crys-

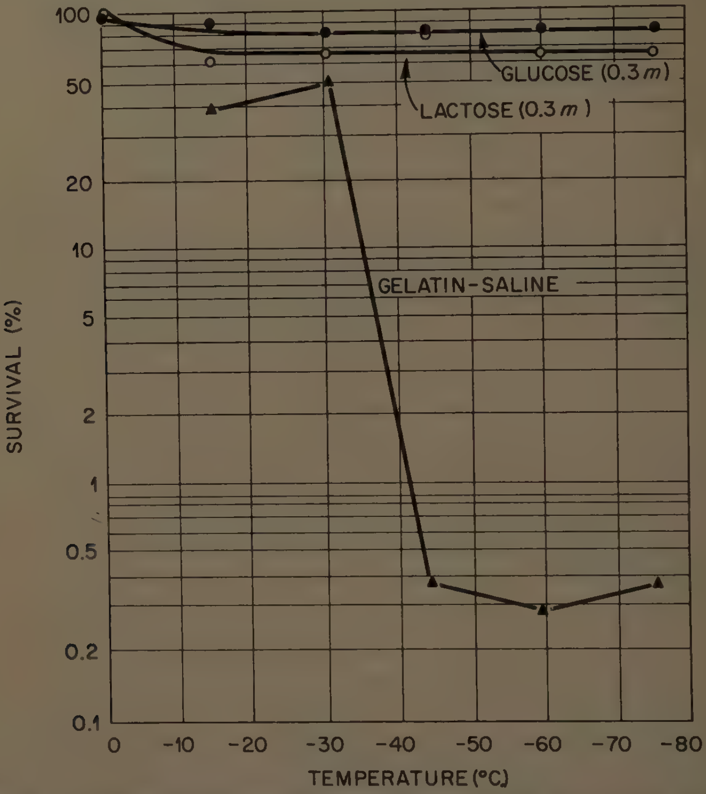

FIGURE 8. Percentage of *Pasteurella tularensis* cells surviving slow cooling to indicated temperatures and subsequent slow warming while suspended in various solutions.

TABLE 5

SURVIVAL OF *ASPERGILLUS FLAVUS* SPORES IN 40 PER CENT ETHYLENE GLYCOL OR IN WATER AFTER RAPID COOLING TO −65° C. AND SLOW WARMING

Suspending medium	Mean percentage germination
40% Ethylene glycol (frozen)	93.5
Water (frozen)	3.8

tals from distilled water readily penetrated a 0.05-mm. hole in an otherwise impermeable polystyrene sheet.[21] However, ice crystals from 0.1 M sucrose did not penetrate a 0.5-mm. hole, and ice crystals from 0.1 M potassium chloride did not penetrate a 3.0-mm. opening. Once again, it must be emphasized that this suggestion that sugar and ethylene glycol afforded protection by preventing intracellular freezing is only a suggestion.

Generalizations and Conclusions

This discussion has been concerned with what happens to the cells of three species of microorganisms during exposure to temperatures below 0° C. We have seen that survival can vary tremendously, depending on the temperatures to which the cells are cooled, the rate at which they are cooled, the rate at which they are warmed, and the nature and physical state of the medium in which they are suspended during exposure. On the basis of these observations it would appear that certain physical events are associated with death, and it has been suggested that intracellular ice formation is the most important.

The question now arises as to whether these findings and their interpretations have any general significance and implications, but the answer to such a query would depend on what is meant by the phrase general significance. One could ask, for example, whether the response of the three cells to factors such as various cooling rates is similar to the response of other microorganisms or of other animal or plant cells. The answer could be yes, no, or perhaps, since all conceivable responses to these variables have been reported in the literature. There are cells that survive rapid cooling better than slow; slow cooling better than rapid; or both equally well or badly. There are cells that survive exposure to any subzero temperature, to no subzero temperature, or to some subzero temperatures. There are cells that survive rapid warming better than slow; slow warming better than rapid; or either equally well or equally badly. Some are protected by sugars or ethylene glycol, others are not. Some are killed immediately at subzero temperatures, others survive indefinitely, and still others survive at some temperatures but not at others, in some suspending media or not in others. On the basis of this sort of chaotic jumble of agreeing and conflicting findings, one can hardly generalize from one strain of a species to another strain of the same species, much less generalize across families, classes, phyla, or kingdoms.

Part of the difficulty arises because the information in most studies is not sufficiently complete to determine the degree to which differences in response to low temperatures result from inherent differences among organisms or from differences in the procedures followed in effecting low-temperature exposure. Moreover, in many cases the information is not sufficiently complete to permit one to deduce the basic factors that were responsible for death.

Perhaps it would be more meaningful to ask whether the physical factors implicated in the death of the three microorganisms considered here are also implicated in the death of other cells and organisms. It was noted, first, that the microorganisms were not harmed by low temperatures when ice formation was absent. This seems to be true for most cells, but not for all of them. Mammals, for example, can be killed by cooling even in the absence of any ice formation,[5] and so can some plants.[6] This phenomenon, which is sometimes referred to as thermal shock, is also held to be the basis of the harmful effects of rapid cooling to subzero temperatures on some mammalian cells, especially sperm.[10,31] Most of the latter studies, however, have not experimentally distinguished the effects of temperature itself from the effects of ice formation, and it is therefore by no means clear that thermal shock was actually the cause of the deleterious action of rapid cooling.

Although ice formation was a prerequisite for death of the microorganisms at low temperatures, the key prerequisite appears to have been ice formation within the cell. Most investigators believe that the formation of intracellular ice is invariably lethal, but in the present case the great effects of rate of warming have necessitated the conclusion that the formation of intracellular ice may not have been as important a factor in death as what happened subsequently to that ice during warming. Perhaps the basic factor is not whether ice is present within cells, but the size of the ice crystals.

There are even some suggestions that the lack of injury observed with the technique of ultrarapid cooling may be due more to the small size of the resulting internal ice crystals than to the complete avoidance of ice crystals by vitrification.[32,33] For one thing, it is probable that many ultrarapid cooling techniques do not produce sufficiently high rates to yield vitreous water. Stephenson has estimated that a cooling rate of 5000° C./sec. is needed, or that a piece of tissue must be cooled to below $-100°$ C. in 0.02 sec.;[34] moreover, even when glassy water is produced under the most rigorous laboratory conditions, it is apparently only partially amorphous.[35,36]

Ultrarapid cooling is believed to be protective only if the rate of subsequent warming is also ultrarapid.[32,37] If the protective action of ultrarapid cooling turns out to be in fact a matter of crystal size, then the lethality resulting from lower warming rates would also be a question of the size of crystals rather than of their absence or presence; that is to say, the lower warming rates could permit the microcrystals to grow to destructive size by migratory recrystallization.[25] Indeed, Rey has actually observed such destruction during the slow warming of rapidly cooled fibroblasts.[38]

Between the matter of death in the absence of freezing and death from intracellular freezing lies the question of the effects of extracellular freezing. In the case of *Aspergillus*, *Pasteurella*, and *Saccharomyces* it seems that extracellular ice formation is not especially harmful in itself, but that it is a prerequisite for intracellular ice formation which may be harmful. In seeking to determine whether extracellular ice formation exerts a similar role in other cells, one is faced with the fact that generally investigators have not attempted to distinguish experimentally between extracellular and intracellular freezing. There is, nevertheless, abundant evidence to show that many living cells can survive with a high proportion of their water frozen, ignoring the question whether that water freezes inside or outside the cells. Wood, for example, has shown this to be true for yeast,[39] and Kanwisher has shown it for molluscs.[40]

It is equally clear that there are many cells that cannot survive extensive extracellular freezing, and several theories have been advanced to account for the cause of damage. The consensus is that injury does not result from the purely mechanical consequences of ice formation.[41,42] Rather, it is widely held that extracellular freezing is harmful because it exposes cells to the high concentration of solutes in the residual unfrozen solution.[10] In other words, freezing is said to be harmful because it effectively removes water from the extracellular solution and from the cells themselves. In a sense, death from freezing is thought to be equivalent to death from dehydration.[33] If this concept is restricted to mammalian cells, the evidence in its support (especially that contributed by Lovelock[30,43]) is convincing. However, it is not a convincing explanation of the role of extracellular freezing in the death of the three microorganisms, for reasons that have already been discussed. As a matter of fact, the distinction between death from dehydration and death from ice formation is very clear with *Aspergillus* spores, for the spores survive dehydration alone much better than they survive dehydration accompanied by freezing temperatures. Ordinary freeze-drying or lyophilization is decidedly harmful to the spores, whereas dehydration in the absence of freezing (by spray drying or desiccator drying) is comparatively harmless.[15,44]

Perhaps the inability to state conclusions of general significance is a reflection of ignorance in two major areas of low-temperature biology. One of these areas concerns the question of what sort of damage is inflicted on cells that are killed by exposure to low temperatures. Is the damage gross crushing and tearing, or is it subtle? Is it structural or functional? Is it reversible or irreversible? Is it cytoplasmic or nuclear? What is the relationship between the physical events implicated in death and the observed damage that accompanies death? Answers to such questions as these are, at best, fragmentary and, at worst, totally lacking.

The second area of ignorance relates to the question of why different cells differ so greatly in their susceptibility or resistance to low temperatures, for the range of susceptibility is strikingly broad. What biological factors are involved? Cell size? Water content, or degree of water binding? Chemical composition or biochemical processes? What is the nature of resistance? Is it predominantly a matter of a cell's having structural and chemical characteristics that prevent the occurrence of lethal physical events such as intracellular freezing? Or is resistance attributable to the ability of a cell to tolerate the stresses that result from the events? Or to its capability to repair whatever damage is inflicted by the events?

The more we learn of the physical factors implicated in death, of the nature of the cell damage leading to death, and of the biological factors that preclude death, the better we should be able to put low-temperature biology on a more rational basis than it now enjoys. There is even the possibility that, as we become more aware of the factors involved in death at low temperatures, we shall find low temperatures becoming useful tools for investigating the physical and chemical factors that determine cell structure and control cell activity at normal temperatures.

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Part III. Freeze-Drying

PRINCIPLES OF FREEZE-DRYING

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When a tissue or other hydrated biological material dries from the liquid state it suffers severe shrinkage, is often altered chemically, and becomes relatively insoluble. When preservation for subsequent use is desired, one or more of these changes renders the final product unusable. However, it is possible to circumvent these undesirable consequences of drying by carrying out the removal of water from the frozen rather than the liquid state. The many considerations involved in the initial freezing of biological material are discussed thoroughly elsewhere in this monograph. It is the purpose of this presentation to examine the mechanism by which the drying of a frozen specimen takes place and the practical techniques by which this can be done.

Freeze-drying, in brief, is the removal of water from a specimen after it has been frozen.[1-3] As opposed to the destructive effects of drying from the liquid state, freeze-drying, under proper conditions, permits the maintenance of three major specimen characteristics: morphology, solubility, and chemical integrity. These characteristics are individually discussed below.

Morphology. When a specimen is frozen, the free water is removed from it and concentrated into scattered ice crystals composed of pure water. The size and distribution of these crystals will depend upon the speed of freezing.[4] Since the water is not actually removed from the specimen, there is no gross change in volume other than the slight expansion resulting from the crystallization of ice. If the water is then removed from the ice directly to the vapor phase by one of the techniques described below, the specimen may retain its structure exactly as it existed in the frozen state, save that the positions once occupied by ice crystals will be simply empty holes. Where preservation of gross structure only is desired, large ice crystals derived by slow freezing can be tolerated. Where histological integrity on the microscopic level is the goal, freezing must be as rapid as possible in order to minimize the extent of ice crystal artifacts.

Solubility. Loss of solubility following drying from the liquid state can be blamed in part upon chemical alterations of the specimen, but probably to a greater extent upon reduction of effective surface area. A freeze-dried material that contains the many spaces remaining after sublimation of the ice crystals has a spongy structure and an effective large surface-to-volume ratio that facilitates resolvation. The freeze-drying of many pharmaceuticals is carried out primarily to obtain a readily soluble product.

Chemical integrity. As water is removed from a specimen, all of its solutes are concentrated at least to a saturated solution. This is obviously not a suitable milieu for many biological compounds or systems. Although freezing creates a similar dehydration and concentration, rapid denaturation can be avoided by virtue of the reduced reaction rates obtained at low temperature. Consequently, if one can freeze a specimen without destroying the system to be preserved and can dry it at reduced temperature before denaturation has occurred, biochemical systems and even living entities can be maintained intact.

Principles of Freeze-Drying

The process of dehydrating a frozen specimen can be divided into three steps: (1) the introduction of heat to supply the energy necessary for sublimation, (2) the transfer of water vapor from the subliming ice crystal through the already dried shell of the specimen, and (3) the removal of water vapor that reaches the surface of the specimen. These three steps in the drying process are analyzed in detail below.

Heat input and sublimation. An ice crystal is composed of pure water rather rigidly confined in a crystal lattice. Within the restrictions of its position, the water molecule continues to indulge in random thermal motion. Statistically, there is a certain probability that a molecule may, as the result of this thermal motion, pop out of its hole and become free of the restraints of the crystal. The possibility that a molecule will leave its position in the ice crystal depends upon the degree of violence of its random thermal motions and the expectation that a particularly violent motion will project it past the energy barrier that restrains it. The violence of the random motions is a function of temperature; in fact, this is a kind of definition of temperature. With a reduction in temperature the thermal agitation of the water molecule is less, and the prospects that it may leave its position diminish.

The transition from the solid to the vapor is a transition from a restricted to a relatively unrestricted state, with an according increase in the amount of energy possessed by the molecule. This additional energy, the latent heat of sublimation, is acquired by each molecule that makes such a transfer of state. The energy itself is derived primarily from the environment in the form of heat. Thus, as each molecule transfers from ice to vapor, a discrete amount of heat is removed from the environment and, unless it is replaced from without, the temperature of the environment will fall. A temperature reduction in turn serves to reduce the probability of further sublimation. The end result of such a closed cycle is the attainment of a temperature so low that sublimation cannot take place.

If heat is introduced into the system at a fixed rate, it will support continuing sublimation at a fixed rate. The temperature of the drying boundary, in such case, will be self-determining. If sublimation takes place at a rate more rapid than the supply of heat will support, the temperature will fall until a level is reached at which the rate of sublimation is exactly equivalent to that which the heat input can maintain. Conversely, if the temperature is too low, the sublimation rate will be less than that which the incoming heat could support, there will be an excess of incoming heat, and the temperature will rise again until sublimation exactly equals that supportable by heat input. Drying-boundary temperature, then, is a variable that depends upon the rate at which heat is provided.

In the foregoing discussion we have assumed that all subliming water molecules have been promptly removed from the system. In actual fact this is not the case, and a second factor that influences specimen temperature must be considered.

When sublimation from ice takes place within a closed container, an increasing concentration of water vapor develops in the space around the ice. This creates an opportunity for water vapor molecules to return to the ice crystal should they happen to strike a suitable site with the proper energy and orientation. Obviously, the higher the concentration of vapor molecules surrounding the ice, the greater the possibility that a return will take place. Each time a vapor molecule returns to the solid state, latent heat is returned to the environment exactly as it was acquired during sublimation. Eventually, an equilibrium will be established between the subliming and recondensing vapor, provided there is no introduction or removal of heat from the system. If heat is introduced, the temperature of the specimen will rise, the sublimation rate will increase, and the concentration of vapor molecules in the space will increase. This in turn increases the rate at which molecules return to the crystal, and a new equilibrium will be established at the higher specimen temperature with an increase in surrounding vapor concentration.

In practice, then, specimen temperature is determined by the rate of heat input and the rate of vapor transfer away from the drying boundary, since these factors reflect the rate of actual heat utilization.

Water vapor transfer away from the drying boundary. Under conventional freeze-drying circumstances an increasing shell of dried material develops as the drying boundary progresses into the specimen. All water vapor produced by sublimation at the drying boundary must pass by diffusion through this barrier of increasing thickness. As a concentration of water vapor moleclues develops at the drying boundary, diffusion toward an area of lesser concentration will take place. The coefficient of diffusion, which is a measure of the degree of obstruction introduced by the dried shell, is not an easy figure to predict. The shell may have cracks, vascular pathways, or holes left by sublimed ice crystals that provide intermittent low-resistance pathways. However, regardless of the exact nature of this resistance to diffusion, it is very high. Stephenson,[5] in a very thorough analysis of the subject, estimates that a dried shell 1 mm. thick introduces a reduction in drying rate "by a factor smaller than 0.001 relative to the maximum rate of sublimation of ice at the same temperature."

It is imperative to recognize that the only force that drives water vapor from the drying boundary to the specimen surface is a concentration gradient. One often encounters the intuitive feeling that, when the specimen is placed in a vacuum chamber, the vacuum will tend to "suck" water out of the specimen. Nothing could be further from the truth. The only effect of an external vacuum on circumstances within the dried shell will be to reduce the concentration of gas molecules present in interstices of the tissue and thus somewhat to reduce the resistance to water vapor flow. The water vapor itself is diffusing along a concentration gradient that is the sole motivation for its net transfer.

Let us now consider a hypothetical situation that will recapitulate the interdependence between heat input, specimen temperature, and drying rate. Assume a frozen specimen at very low temperature, contained in any proper freeze-drying apparatus. As heat is supplied, the surface temperature of the specimen rises, and sublimation begins. A temperature is approached at which the sublimation rate will be comparable to the rate of heat input. However, as drying progresses, a layer of dried material develops, and an increasing resistance to vapor flow is introduced. This situation leads to an increasing concentration of vapor at the drying boundary, with an increasing amount of vapor returning to the ice crystal and giving up its latent heat. This effect reduces the net amount of heat utilized, and the available excess produces a rise in temperature. With increasing temperature, the sublimation rate increases, a higher vapor pressure develops at the drying boundary, the diffusion gradient to the surface steepens, and vapor diffuses to the specimen surface at a greater rate until the rate of vapor removed corresponds again to the rate of heat input. During subsequent drying an equilibrium is constantly being approached between heat input, sublimation rate, recondensation rate, vapor pressure, and vapor flow. In the last analysis it will be vapor flow that limits the rate of drying for, as shown by Greaves' experiments, described elsewhere in this monograph, the rate at which heat can be utilized for sublimation appears to be almost limitless provided there is no obstruction to vapor removal.

This analysis also demonstrates the continuing change of circumstance as drying proceeds and the dried shell increases in thickness. One of the major problems in both experimental and commercial freeze-drying is our ignorance of the exact state of these changing conditions within the specimen during drying.

Reduction of vapor pressure at the specimen surface. Since the only motive force to effect a transfer of vapor across the dried shell is a water vapor concentration gradient, the steepness of this gradient will be a major factor in determining the rate of vapor transfer. The gradient can be increased by raising the concentration of vapor at the drying boundary but, since vapor pressure at this location is directly related to temperature, there is a limit to which the vapor pressure here can be increased without developing too high a temperature at the drying boundary. The gradient can also be steepened by reducing the vapor pressure at the specimen surface to a minimum; much of the design of freeze-drying equipment is directed toward this goal. The ideal, of course, is to maintain a zero vapor pressure around the specimen. This would require that each molecule that reached the surface could continue on without impediment, so that no congregation of vapor molecules would remain surrounding the specimen to constitute a finite external vapor pressure.

The ideal vacuum freeze-drying system would consist of a specimen suspended freely in outer space, receiving heat by radiation uniformly on all sides. In actual practice this goal can only be approached, not attained, since specimen supports and other necessary instrumentation constitute obstacles to the straight-line escape of water vapor molecules. In addition, the evolution of water vapor from the specimen may fill the surrounding space and, in effect, can constitute an obstacle to its own diffusion. The function of the vacuum system in vacuum freeze-drying is simply to provide as long a mean free path as possible, so that the transfer of water vapor away from the specimen surface is as efficient as possible with the minimum number of collisions en route.

Freeze-Drying in Practice

The following section will consider separately the various practical approaches to the three aspects of freeze-drying discussed above.

Heat input. The most common manner in which heat is introduced to a drying specimen in commercial apparatus is from a heated surface upon which the specimen rests. Heat is delivered to the drying boundary by conduction through the frozen mass of the specimen, with the evolution of vapor taking place from its opposite side. FIGURE 1 shows schematically the limitations of this approach. Since heat can flow only down a thermal gradient, the amount of heat that can be delivered to the drying boundary will be determined by the thermal conductivity of the frozen material, the temperature of the drying boundary, and the temperature of the specimen surface adjacent to the heated support. The thermal conductivity of the frozen material is relatively low for most biological materials. Mammalian tissue in the frozen state has, for example, a thermal conductivity roughly 3 per cent that of copper.[6] The temperature of the drying boundary, as shown previously, depends upon the amount of heat supplied, the rate of sublimation, and the effectiveness of vapor removal, and it is only indirectly controllable. The temperature of the surface adjacent to the heated support is under the immediate control of the operator, but this temperature is seriously limited, since there is always a maximum safe temperature for the frozen material in question. In some cases this will be its melting point; in others it may be necessary to maintain a much lower temperature to prevent biochemical alterations. Thus there is a severe limitation upon the amount of heat that can be made available at the drying boundary. Furthermore, it will always be less heat than can be utilized. For example, as in FIGURE 1, if the maximum safe temperature of the tissue is −10° C., one would like to have the drying boundary at this temperature in order to develop there the maximum water vapor pressure to produce the maximum concentration gradient for vapor transport to the outside. However, since the temperature adja-

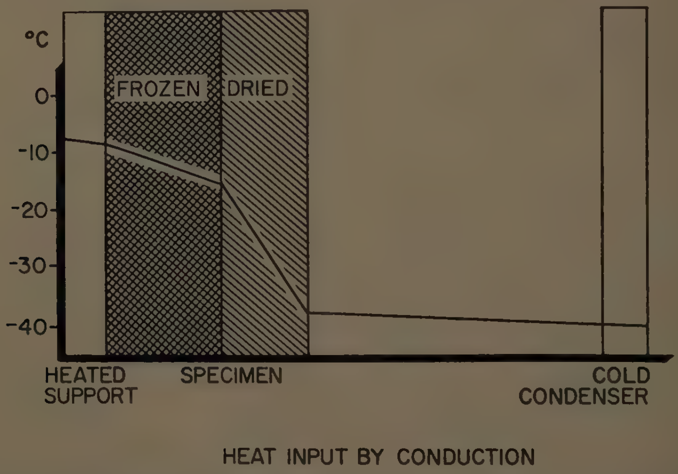

FIGURE 1. The character of temperature gradient to be expected when drying is supported by conductive heat input. In this example the temperature of the frozen specimen cannot exceed −10° C., which is the limit to specimen temperature at point of contact with the heated support. Since heat can flow only down a gradient, the temperature at the drying boundary can be only lower. The actual temperature at the drying boundary will depend upon the rate of vapor transfer out through the dried shell (that is, the rate of energy utilization) and the thickness and conductivity of the frozen portion. In any case, drying will always take place at less than maximum permissible temperature.

cent to the heat source must always be warmer than the boundary in order to develop a gradient, the temperature of the boundary is always less than the optimum. This method of heat supply also suffers from the disadvantage that drying must not take place from the side of the specimen that is in contact with the heat source, otherwise the conductive path will be destroyed. If any drying does take place at this surface, the dried shell produced will create a high-resistance barrier to heat flow. Unless the internal temperature of the specimen is monitored, the operator may be unaware that the resistance to heat input has increased by a factor of 10 or more. Even if he is aware of this, the operator is powerless to know how to control heater temperature for maximum heat transfer without exceeding permissible temperature elsewhere in the specimen.

A second approach to heat input is by radiation from infrared sources. There is one immediate benefit to be gained by this approach, since a specimen can be suspended in the drying chamber and radiant heat delivered to it from all sides. Drying a flat specimen from both sides instead of from a single side, as in conduction heating, improves the drying rate roughly by a factor of 4. Radiant heating, however, is still far from an ideal solution, since it is still necessary for the heat to traverse a thermal gradient between the surface and the drying boundary. Furthermore, the thermal insulation provided by the dried shell in a vacuum is considerable, so that the gradient must be quite steep in order to deliver heat through any appreciable thickness of dried shell. Again, the limit to the steepness of this gradient will be the maximum permissible temperature at the specimen surface that cannot be directly measured. If the surface temperature rises too high, charring may take place. FIGURE 2 shows the thermal gradients to be found in a typical example of radiation heating. It may be seen also that this type of heat input has the advantage that it does not raise the temperature of the frozen interior.

Heating by radiation has one other disadvantage, particularly in commercial applications. In order that heat may be transferred, the specimen must be in a direct line of sight from the source of heat. This means that if multiple specimens are used they may be shadowing each other so that the benefits of heating from all sides are not experienced. This is particularly true in applications where specimens such as peas or beans are tumbled in a warm drum during drying. At any given moment the majority of the particles are receiving no radiation from the walls, and drying is far less efficient than it might be.

A third method of heat input has been frequently proposed and occasionally attempted.[7] This is the introduction of energy through very high-frequency electromagnetic radiation in the microwave range. Wave lengths in the region of 12 cm. are most generally used because high-energy commercial equipment is available in this range. At first glance it might appear that this method offers an excellent solution to the problem, since the dried shell is transparent to energy of this wave length, and energy presumably is uniformly absorbed by the frozen interior. FIGURE 3 shows the sort of thermal gradients to be ideally expected using microwave radiation. Since, theoretically, the energy is uniformly introduced into the frozen interior, the heat developed in central portions must still flow to the drying boundary by conduction along a thermal gradient. This means that the center of the specimen will be at a higher temperature than the drying boundary, and it is still impossible to introduce the maximum usable amount of heat to the drying boundary. This approach, then, is comparable to conduction heating, save that drying may take place from all sides, and there is a slight improvement in the ability to deliver heat to the boundary. On the other hand, no direct control is possible in routine practice. In the absence of a temperature-measuring element at the center of the specimen read intermittently with the microwave power off, there is no way of know-

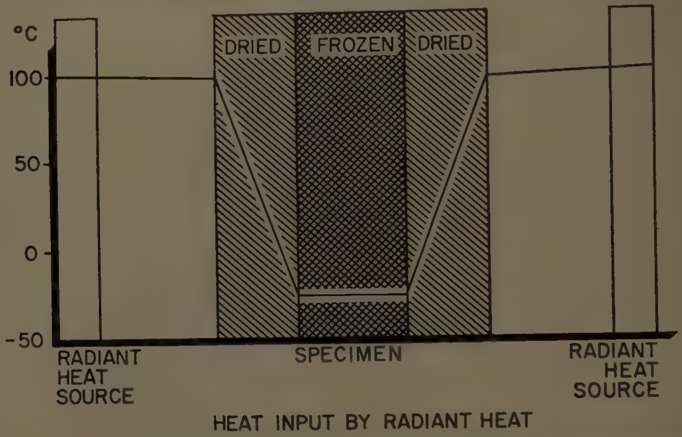

FIGURE 2. The temperature gradient during radiant heating. Since heat is introduced through the dried shell, maximum permissible temperature at the surface is much higher, usually limited by charring. However, the thermal conductivity of the dried shell *in vacuo* is very poor, and heat transfer is seriously restricted despite the high external temperature. The advantages of radiant heating over conductive heating are that a low temperature of the frozen interior is maintained, and drying from all sides is possible. In addition, as the rate of vapor transfer falls off with increasing thickness of dried shell, heat input also is reduced for the same reason, providing some degree of built-in compensation.

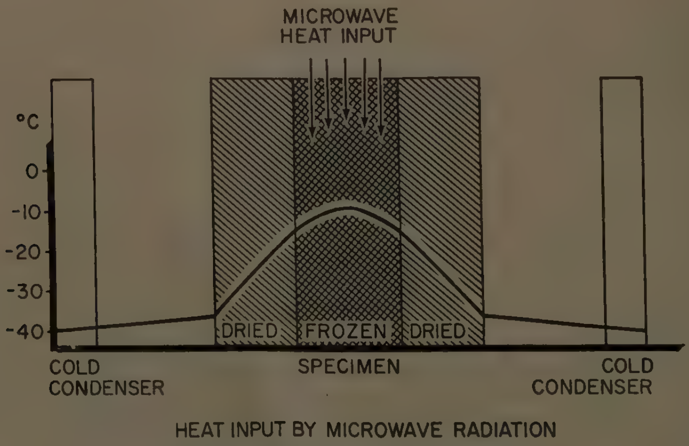

FIGURE 3. Theoretically, heat, when introduced by microwaves, is uniformly distributed throughout the frozen interior. This still requires conductive heat transfer, since energy introduced in the frozen portion must pass by conduction to the drying boundary. Heat input is limited by the maximum permissible temperature of the frozen material, and the drying boundary temperature must always be less than the maximum permissible.

ing what the internal temperature is. This is a more serious problem than might at first appear, since the absorption of microwave energy by liquid water is several thousand times greater than that by ice. This means that, if the internal temperature should anywhere rise to the melting point, the absorption of energy at this spot will suddenly become vastly greater. Pockets of steam can be created in this way, with explosive results. This positive feedback situation is compounded by the fact that, in practice, microwave absorption is not completely uniform, and hot spots can develop as a result of variations in geometry and dielectric of the specimen.

It is apparent that ideal means for the introduction of heat to the drying boundary have not been developed. Although ultimately the rate-limiting factor in drying is vapor removal, the drying boundary should at least be at the maximum permissible temperature. This will in turn provide the maximum vapor pressure at the boundary and increase the concentration gradient and vapor flow to the surface.

Vapor transfer across the dried shell. There appears to be very little in the way of tricks that can be devised to decrease the resistance to diffusion of water vapor through the dried shell. Procedures such as drying from all sides and reducing specimen thickness are self-evident. The device of punching holes into the specimen to provide pathways for vapor transfer has led to an increase in the drying rates for meat. It is generally felt that a freezing that produces large ice crystals is also advantageous in producing low-resistance pathways within the tissue. At present, however, it appears that dried-shell resistance is unavoidable, and that efforts can be directed only toward increasing the concentration gradient across this barrier by raising the vapor pressure at the drying boundary and reducing it at the surface.

Reduction of vapor pressure at the specimen surface. Until very recently the only method of removing water vapor once it had reached the surface of the specimen was by transfer through an evacuated space to a condensing surface. The condensing surface is generally created by low temperature. Chemical desiccants or adsorbants have been used, but these materials suffer from the fact that their surfaces may become saturated while deeper portions are prevented from functioning because of their location. In some systems no trap is used, and the vapor is carried out through the vacuum pumps. There are two criteria that must be observed for efficiency in this phase of drying:

First, the transfer of water vapor from the specimen surface to the condenser must be efficient; this is the function served by the vacuum system. At atmospheric pressure, collisions between water and air molecules are so numerous that diffusion is a relatively slow process. As the pressure is decreased, the average distance that a molecule will travel without collision increases and, in good vacuum systems, this distance can exceed that from specimen to condenser.

Second, the condensing surface must be so placed that it subtends as much of the field of view of the specimen as possible. Ideally, the condensing surface should surround the specimen completely so that all vapor molecules striking the chamber walls will be removed from the system. Such a design is illustrated diagrammatically in FIGURE 4. In this device* the entire specimen chamber is immersed in liquid nitrogen and thus comprises a completely surrounding condensing surface. Such an apparatus, operated at high vacuum, provides very nearly the optimum conditions for vapor removal. An opposite extreme is that best illustrated by a system in which the condenser is at the end of a long tube, or in which the vapor is removed through the vacuum pump. In these two instances the condensing surface is, in effect, at best, the cross-sectional area of the port leading from the drying chamber. Vapor molecules striking this opening may be removed from the system; those striking any other surface must rebound until by chance they enter the orifice leading to the trap or pumps. It should be noted that the importance of this factor depends

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* Manufactured by Canal Industrial Corporation, Bethesda, Maryland.

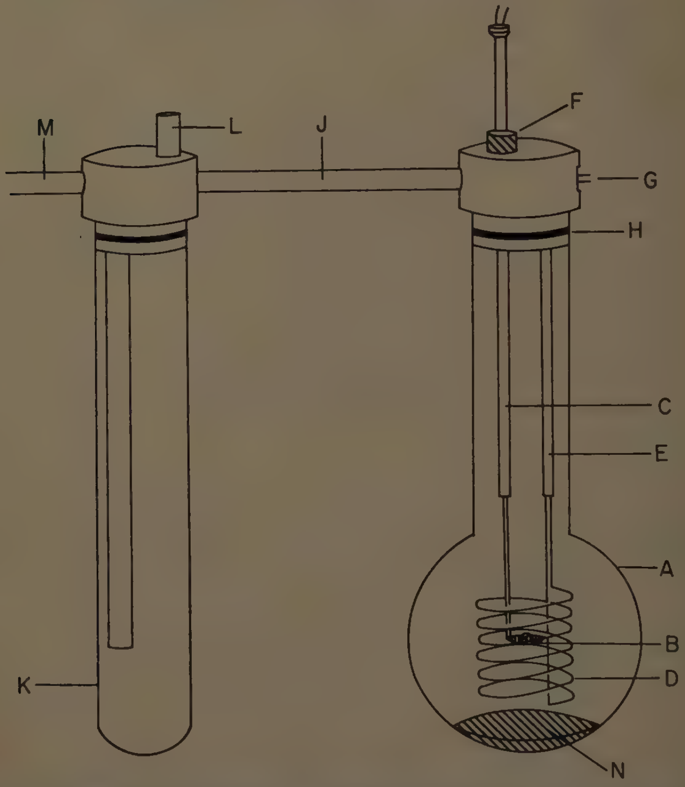

FIGURE 4. Freeze-drying apparatus. Maximum efficiency of vapor removal is provided by immersion of the entire vacuum drying chamber (A) into liquid nitrogen or dry ice-alcohol solution. Complete flexibility of drying temperature is possible. The temperature of the specimen's radiant environment is estimated by measuring the temperature of the specimen support screen (B) with a thermocouple. A feedback circuit then controls the radiant heat output from the heater coils (D) to maintain desired specimen temperature. On the completion of drying, the liquid nitrogen bath is transferred to surround envelope (K), which now serves as a conventional cold trap to prevent vapor released from the drying chamber from reaching the vacuum pumps, which are connected to the system at M. Paraffin (N) is melted by the external application of heat, lock nut (F) is loosened, and the specimen support rod (C) can be lowered until the specimen is immersed in the paraffin. In this manner embedding may be carried out without breaking the vacuum. A vacuum gauge may be attached at the port (L).

greatly upon the actual rate of sublimation of the specimen.[8] If the sublimation rate of the specimen is extremely low, the restricting effect of a small orifice to the trap will be reduced. Since most large-scale freeze-drying systems operate at a relatively poor vacuum where the mean free path is an infinitesimal portion of the specimen-to-condenser distance, due both to the air pressure and high concentration of water vapor, condenser location and area can be of considerable significance.

An alternate approach to water vapor removal has been recently reported[9] in which water molecules reaching the specimen surface are swept away by a flow of dry air at atmospheric pressure. There are several apparent advantages to this approach. In the first place, the elimination of the vacuum-tight system and the vacuum pumps is a significant gain. Desiccation of air at atmospheric pressure can be carried out more conveniently and economically with reusable adsorbants than by use of refrigerated condensers. One interesting aspect of the atmospheric pressure system is the greater degree of control over drying temperature. Heat transfer to the drying boundary will be by conduction through the dried shell and will thus be comparable to heating by radiation (FIGURE 2). As opposed to radiant heating, however, the temperature of the dried shell surface will be known exactly, since it will be the same as the air temperature. Furthermore, since the air flows around all surfaces of all specimens, heat input will be uniform and there will be no effect of shadowing as in radiant heating. There will also be a modest improvement in the thermal conductivity of the dried shell at atmospheric pressure as opposed to the situation *in vacuo*. By the proper choice of desiccant it is also possible to remove water selectively. If, in a closed system, the air is saturated with other volatile components, these will not be removed from the specimen as they would in a vacuum system.

The disadvantage of the atmospheric pressure technique lies primarily in the increase in resistance to vapor flow in the dried shell. Holes, cracks, and other interstices within the dried shell no longer provide low-resistance paths for vapor transfer, and a reduction in drying rate is experienced from this cause. The drying of mammalian tissue at −30° C. carried out at atmospheric pressure is reported to take approximately twice as long as in the high-vacuum system illustrated in FIGURE 4.[9] There are no reports as yet on the value of this system in higher-volume, higher-temperature applications where the operation of vacuum systems is much further from ideal.

An alternate approach to sublimation freeze-drying that should also be mentioned is liquid substitution.[10] This technique requires that the frozen specimen be immersed in a dehydrated liquid that has a eutectic point with water at a temperature lower than that at which the freeze-substitution is carried out. Both alcohol and acetone are commonly used. One of the advantages of this system is that the removal of ice can take place at a low temperature at which the vapor pressure would be too low to permit sublimation drying in a reasonable period of time. Even so, the substitution of solvent for ice usually requires many days or weeks. Its principal value is in the preparation of histological specimens.

It is hoped that the foregoing summary of the general principles of freeze-drying will aid in indicating both the virtues of this technique and its inadequacies. Some of these inadequacies may be overcome by new techniques, with a resulting increase in the efficiency and speed of drying. The experiments described by Greaves elsewhere in this monograph illustrate the tremendous drying rates possible at very low specimen temperatures if the barriers to heat and vapor transfer can be overcome. Such increases in drying rate at low temperature will not only permit broadened industrial applications, but should make more practical the preservation of living cells, to which end rapid drying appears to be an essential element.

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THE THEORY AND PRACTICE OF FREEZE-DRYING

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Introduction

Drying is the traditional method of preserving natural substances containing water, in the absence of which many chemical, physical, and enzymatic changes are arrested or retarded. However, such changes may take place during drying. Those that occur in foodstuffs dried in the conventional manner are familiar and may yield, as in the case of sun-dried figs or raisins, a new product that need not be compared with the fresh fruit. There is, however, an increasing demand for food preserved in such a way that it can be restored to its natural condition. Such dietetic sophistication commands our attention less than the needs of medicine and surgery for bacterial cultures and human arteries preserved as perfectly as possible.

When aqueous protein suspensions or solutions, or wet solids containing salts and protein, are dried there is a movement of the solid constituents to the surface of evaporation, where they form an increasing concentration as drying proceeds. Often a dense impermeable skin is formed that can inhibit further drying and may be insoluble. In the case of proteinaceous tissue some of the structural protein may also dissolve in the highly concentrated salt solutions that form near the surface. Considerable over-all shrinkage may occur that further reduces the solubility, and the viability of the tissue may be lost. Similar effects are observed in the drying of suspensions of microorganisms.

These effects are not much reduced by drying under vacuum, although this does permit the use of lower temperatures without, necessarily, any loss of speed of drying.

A well-established method of preservation is deep-freezing but it is not always realized that the process is one of dehydration, although the ice crystals that form are not removed bodily from the other solids present in the frozen mass. However, changes can occur under conditions of deep freezing. Greater disadvantages are the cost of frozen storage and transport and the chance of refrigerator failure, which would spoil the goods. Dehydration involving loss of weight can occur during frozen storage—a problem that vendors of frozen food are now having to face—and special techniques are sometimes required for thawing, the need for which in any case prevents immediate use of the product.

Many of these objections are minimized and sometimes eliminated by drying from the frozen state. This process, known as freeze-drying, consists in the sublimation of ice from frozen suspensions, solutions, and water-containing solids, leaving a dry spongy mass of about the same size and shape as the original frozen mass, having improved heat stability and easily redissolvable in cold water. When used to preserve heat-sensitive, vital substances, the method can ensure a minimum loss of viability, potency, or vitality when reconstitution takes place immediately after drying, with negligible further loss when reconstitution is carried out only after long storage at or above room temperature. Solubility is higher than that existing after other methods of drying, and that of an already dry substance may often be increased by redissolving and freeze-drying it. Under the proper conditions, all biological activity is arrested by the drying process.

The ease and accuracy with which fluids can be dispensed, as compared with powders, is well known, and it is sometimes an advantage to redissolve substances prepared in the dry state by precipitation or other means, in order to put them into individual containers and then to freeze-dry them. The process also achieves what is picturesquely called "pharmaceutical elegance."

Where arteries, skin, or bone for grafting purposes are to be preserved, freeze-drying permits long-term storage at room temperature with immediate availability in an emergency. Depending on the technique used, the physical properties of the tissue may be more or less retained, with some controlled loss of antigenic properties.

With tissue for histological and cytological investigation any form of drying permits the embedding of a specimen in wax or resin for sectioning and staining, but freeze-drying retains the physical structure substantially without artifacts while arresting chemical action and gross migration of the chemical constituents.

When applied to foodstuffs, whether liquid or solid, their long-term preservation at room temperature is ensured by freeze-drying, and many kinds can suffer negligible alteration in flavor, appearance, and texture after reconstitution. The considerable reduction in weight—often exceeding 80 per cent—together with the reduction in bulk by compression or comminution made feasible by drying, can lead to great savings in packaging, storage, freight, and handling charges. The reduction in weight is significant when carriage by air is considered, and it has been calculated that the saving in freight charges by air over a distance of 500 miles can offset the not inconsiderable cost of drying. The short time of rehydration of freeze-dried food compared with that for thawing deep-frozen food is attractive to the pampered housewife, who also favors a method in which rapid hydration in hot water of freeze-dried precooked food allows it to be served immediately.

As further examples of the diversity of materials that can be preserved by freeze-drying, the recent claim of Meryman[1] to have dried whole blood and spermatozoa may be cited. Mushroom spores, human milk, and whole small dead animals and insects for museum display, complete cooked meals, and highly reactive finely divided metallic powders can also be successfully freeze-dried. Hundredweight quantities of newly formed apple fruitlets have been freeze-dried for transportation to the Antipodes for the examination of growth-promoting constituents at a time when they were not in season locally.

As practiced at present, freeze-drying is a batch process. Continuous processing is feasible, but is not discussed here.

The effect of freezing. Some of the undesirable changes in a substance that are to be obviated by freeze-drying may occur during the prior freezing, the general theory of which is outside the scope of this paper. However, apart from the effect of freezing on a substance before it is dried, its physical state when frozen considerably affects the ease and degree of success of the freeze-drying process. For example, under certain conditions, as the temperature of a substance is taken below 0° C. pure water will freeze out until one or a series of eutectic concentrations that freeze below 0° C. are progressively reached. If a sufficiently low temperature is not attained for the whole of the substance to solidify, small pockets of liquid may remain dispersed throughout the frozen mass. During freeze-drying the ice will sublime, its surface moving away from the outer boundary, leaving behind a number of small vacuoles. Water vapor from the frozen eutectic mixtures will diffuse into and through these vacuoles and finally escape through the surface of the material. Localized pockets of unfrozen solution will evaporate in a similar way, but a small amount of liquid may migrate toward the surface. If the proportion of unfrozen material in the whole mass is small, the physical structure of the matrix will not change during drying. Over-all shrinkage will then be negligible, and the dry substance will have the same size and shape as it had when frozen. If, on the other hand, the proportion of unfrozen liquid is high, true freeze-drying is not achieved, shrinkage occurs, and insolubility and loss of vital characteristics may result. It will be seen later also that the structure of the ice crystal formation, which is affected by the concentration and the rate of freezing of the fluid, in turn affects the practicable rate of drying.

The mechanism of sublimation. The sublimation of pure ice and the evaporation of water vapor from frozen eutectic mixtures, wherein lies the principle of freeze-drying, occur when the partial pressure of water vapor at a frozen surface exceeds that at points close to it.

In an environment of still air at pressures from atmospheric down to a few Torr (refer to the section on measurement of pressure for a definition of units) collisions between evaporating molecules and those of the surrounding air retard evaporation and cause an accumulation of water molecules in the region of the boundary between the solid and the air. As the concentration of evaporated water vapor increases so does the probability that for every molecule leaving the specimen one will return. Equilibrium is reached and drying ceases when, in a closed system, the vapor becomes saturated throughout the enclosure, and any initial temperature difference between the evaporating source and the ambient air then disappears. Complete cessation of drying is avoided when some means of removal of moisture from the system is provided. In these conditions the rate of escape of water vapor from the surface of a specimen at constant temperature may be limited by the speed of diffusion of the vapor through the enveloping air to the moisture-removing agent.

This inhibiting effect may be reduced by the scavenging action of a stream of dry air or gas at atmospheric or reduced pressure[2,3] flowing over the surface of evaporation, or by the maintenance of a low pressure of air above the surface combined with the action of a vapor-removing agent. It is the second method, or vacuum freeze-drying, that forms the subject of the present paper.

Freeze-drying in vacuum. In vacuum freeze-drying the partial pressure of air not only immediately above the surface, but throughout the system, is kept to a value at least ten times lower than the local pressure of the water vapor. The moving environment of air released from solution in the substance being dried and leaking into, and evolving in, the vacuum system, is removed by a vacuum pump. The evolved water vapor may be removed by the vacuum pump unaided or in conjunction with a desiccant or a refrigerated trap.

Low partial pressures of air and water vapor, while providing conditions favorable to drying, do not promote it and, without a supply of latent heat, the rate of evaporation would diminish. The mechanism involved is explained by the kinetic theory of gases. The temperature of a substance is a function of the average speed of the molecules composing it. Molecules having the highest speeds normal to a surface of evaporation are those most likely to escape altogether. When evaporation takes place, the average speed of the remaining molecules is lowered, and the temperature of the aggregate is reduced. It is impossible to isolate a substance entirely from all sources of heat but, in the absence of an adequate supply of energy, evaporative losses cause a fall in temperature of the frozen substance until the increased heat gained from its surroundings, due to the steeper temperature gradient, just balances the reduced rate of evaporation at the lower temperature.

A freeze-drying system may be compared to a refrigerating system in which the refrigerant is water vapor. Heat is absorbed at the evaporator, in this case the product being dried, and the water vapor generated flows down a pressure gradient through restrictions imposed by an increasing thickness of dry material, the container opening, sterility filters, residual air and pipelines, to the point where it and its thermal energy are removed by direct pumping, absorption, or condensation. The comparison is valid, the only real difference between freeze-drying and a conventional refrigerating process being that in the former the refrigerant is not recirculated.

The simplest case for examination is the sublimation of a slab of ice contained in such a way that evaporation can occur from one face only. The following treatment is based largely on the work of Kearsey *et al.*[5]

When the residual air pressure is between 10 and 100 times lower than the vapor pressure of the ice, and means is provided for removing the vapor as it evolves, the rate of sublimation of ice, to a close approximation, is given by

$$G = 60^2\gamma \sqrt{\frac{M}{2\pi RT}}\, P_0{}^{*} \tag{1}$$

where G = rate of evaporation in grams per square centimeter per hour, γ being the evaporation coefficient; M, the molecular weight; R, the gas constant in dyne cm. per mole per degree Kelvin; P, vapor pressure of pure ice at temperature of evaporation; and T, temperature in degrees Kelvin.

Assuming γ to be unity,[6] it should be possible, under the necessary ideal conditions, to sublime at a rate approaching 150 gm. per square centimeter per hour at a surface temperature of 0° C., at which temperature the partial pressure of ice is 4.56 Torr. At the lower temperatures required for solutions the vapor pressure is also lower, being 0.29 Torr at −30° C., and the rate of sublimation is reduced in proportion. Although the presence of residual air and inefficient removal of the vapor reduce the above figure, yet very high rates of evaporation can be achieved if the heat of sublimation is applied where it is required, namely at the surface.

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* P_0 = partial pressure of water vapor at the prevailing temperature.

It is seldom convenient to provide radiant heat to the upper surface of the ice for fear of burning the dry residue that forms above it. Heat is usually applied, therefore, to the underside of the slab through which it is conducted to reach the interface.

Considering again the case of pure ice, uniformly and constantly heated from beneath, the rate of sublimation will be constant and so also will be the surface temperature.

The rate at which heat reaches the surface is given by

$$H = \frac{A\kappa\Delta\theta}{t} \tag{2}$$

where H is the rate at which heat reaches the surface; κ, the coefficient of thermal conductivity of ice in gram calories per square centimeter per degree Kelvin per centimeter per hour; A, the cross-sectional area in square centimeters through which the heat flux is passing; $\Delta\theta$, the temperature drop across the ice slab in degrees Kelvin; and t, the thickness of the slab.

If λ is the latent heat of sublimation of ice in gram calories per gram, the rate of sublimation is given by

$$G = \frac{H}{\lambda} \tag{3}$$

The temperature difference between the upper and lower surfaces will decrease as the slab becomes thinner until, at the moment when the ice disappears, the temperatures equalize. In these circumstances it would be necessary only to control the rate of heating so that the ice in contact with the source of heat did not melt at the beginning of the process.

However, with a frozen solution, the dry layer of progressively increasing thickness left behind as the ice recedes forms a continuously increasing restriction to vapor flow. In order to maintain a constant rate of sublimation under these conditions, the vapor pressure of the ice surface, and hence its temperature, must rise throughout the process. The temperature of the underside of the ice may then remain constant while the temperature of the interfacial surface rises to equal it. This is not necessarily true for all substances of various thicknesses undergoing different rates of drying. It also depends particularly on the extent of the thermal contact with the heat source.

For the easy introduction of heat into the ice, the underside should be in good physical contact with the container wall; otherwise sublimation will occur through the lower face also, and thermal contact with the ice will be lost.

H. Kramers[7] quotes a reported value of the thermal conductivity of ice as 2.4 watts/meter °C., but has found conductivities as high as 3.7 watts/meter °C. in milk frozen at a rate of 0.8 mm./min. and measured in the same direction as the heat flow during freezing. The effective resistance of the dry material depends upon the mode of flow of the vapor through it and the possibility of recapture of an evaporated molecule by an ice crystal.

Practicable rates of drying are almost wholly conditioned by the formation of the dry layer. For solutions they lie between 0.05 and 0.2 gm. per square centimeter of surface per hour.

If the average distance traveled by vapor molecules before they collide with one another (mean free path) exceeds the separation of the walls of the dry material between which they are traveling, the flow is molecular, and the mass rate depends upon the pressure gradient. If the molecules tend to collide with one another before striking the walls the flow is viscous and, by analogy with Poiseuille's equation for flow of a gas through pipes,

$$G = \frac{1}{\rho} \frac{P_1 2 - P_2 2}{\ell} \qquad (4)$$

Where ρ is a constant depending on the material and the viscosity of the gas, P_1 and P_2 are the upstream and downstream pressures, respectively, and ℓ is the thickness of the dry layer.

Kramers has investigated the influence of composition and rate of prefreezing on the permeability of freeze-dried solutions, and he concludes that materials containing less than the eutectic concentration of soluble material yield a structure that permits a short drying time, but that those with more than the eutectic concentration are more difficult or impossible to dry. The content of insoluble material has little effect.

He found that, when concentrations below the eutectic are frozen, under certain conditions flakelike ice crystals separate from the insoluble matter. These may extend through almost the whole thickness of the layer and leave on sublimation comparatively large platelike spaces through which the vapor escapes.

The same investigator expresses the mass fraction of crystallized pure ice as

$$\phi = 1 - \frac{C_s + C_i}{C_e(1 - C_i) + C_i}$$

where C_s = mass fraction of soluble material, C_i = mass fraction of insoluble material, and C_e = mass fraction of insoluble material in pure solution at eutectic composition.

Since ϕ is proportional to F, the free space for vapor flow, the permeability will increase with C_s.

Defining K, the permeability, as

$$\phi = -K\ M\ dp/dx, \text{ with the usual notation;}$$

for flow through parallel slits

$$K = \frac{2}{3} \frac{Du}{RT} \cdot F$$

where u = mean molecular velocity in meters/sec., and R = gas constant, so that $K \sim D\,\phi$ under constant conditions.

When D is constant, the permeability increases as the soluble content decreases.

Kramers emphasizes that the above theory is only qualitative.

The platelike structure could be obtained best with rates of freezing between 0.5 and 1 mm./min. Higher rates reduced the permeability because the average distance between the plates became smaller due to an increase in the number of crystal-generating nuclei per unit surface.

Basic equipment for freeze-drying. A freeze dryer consists in essence of an enclosure in which the partial pressure of air can be reduced by a vacuum pump to 0.05 Torr or less. The enclosure contains a heated shelf on which is placed the product to be dried, also a refrigerated condenser for trapping the evolved water vapor. Means of indicating vacuum, the temperature of the product and the condenser, and of controlling the rate of heating are useful refinements.

It is clear that the temperature of the frozen material depends upon the rate of heating and the partial pressures of air and water vapor in the system, as well as on the thickness of the dry material and other restrictions to vapor flow. Increased leakage of air into the system, failure of the vacuum pump or refrigerator, or saturation of the desiccant can lead to melting of the product unless the heat supply is interrupted.

Simple manifold dryer. The simplest kind of equipment for laboratory scale and pilot freeze-drying consists of a manifold connected to a vacuum pump via a water vapor trap (FIGURE 1). Containers of prefrozen material are attached to the manifold by vacuum unions or hose. The water vapor trap may contain a chemical or a physical desiccant or a surface cooled by solid carbon dioxide or one of the liquid gases. Of the desiccants, phosphorus pentoxide is the most convenient, as it absorbs at least one fourth of its own weight in water before it begins to lose efficiency at the pressure necessary for freeze-drying. Combination with water to form one of a group of phosphoric acids is an exothermic irreversible reaction. Of the refrigerants, solid carbon dioxide, mixed with acetone or ethyl alcohol, is for most purposes equally as effective as the liquid gases, and is considerably cheaper.

The Freeze-Drying of Liquids

The simplest freeze-drying system for routine operation is that for the bulk drying of liquid solutions in trays (FIGURE 2). The trays are supported on shelves that can be heated electrically or refrigerated and are contained in the drying chamber, which is evacuated through a vapor trap by an oil-sealed mechanical pump. The vapor trap consists of a refrigerated coil contained in a water vapor condenser chamber with which the drying chamber communicates.

The degree of vacuum in the system and the temperatures of the shelves, the product, and the condenser are indicated by instruments enabling the course of the process to be observed and controlled.

Prefrozen material, preferably at a temperature of 10° to 20° C. lower than its eutectic, and contained in trays, is placed on the shelves precooled to a similar temperature. The object is to prevent the material from melting before the necessary low total pressure—about 0.5 Torr—has been obtained in the system for evaporation to occur below the eutectic temperature.

The system is closed to the atmosphere and evacuated by a mechanical pump, the condenser coils having been precooled to about $-40°$ C., although substances which must be dried in the region of $-40°$ C. will require a lower temperature for vapor transfer to the condenser to take place. A convenient thickness of material is about 1 cm. In a well-designed system that does not impose its own limits on the rate of drying, this thickness may take from 5 to 20 hours to dry, depending on the nature of the substance, its concentration, and the rate of prefreezing.

When the total pressure in the space between the drying chamber and the condenser has been reduced to 0.3 Torr or less, heat is applied to the shelves, the rate being regulated to ensure that the product does not melt. As the stage is approached when all the free ice has sublimed, as indicated by a fall in pressure to a value approaching the saturation vapor pressure of the ice on the condenser coil, and by a rise in temperature of the product, the heat to the shelves is reduced to limit the final temperature attained by the dry product to 40° C. or below. So-called primary drying is then complete. Secondary drying[8] takes place during an extension of the time already taken in order to reduce

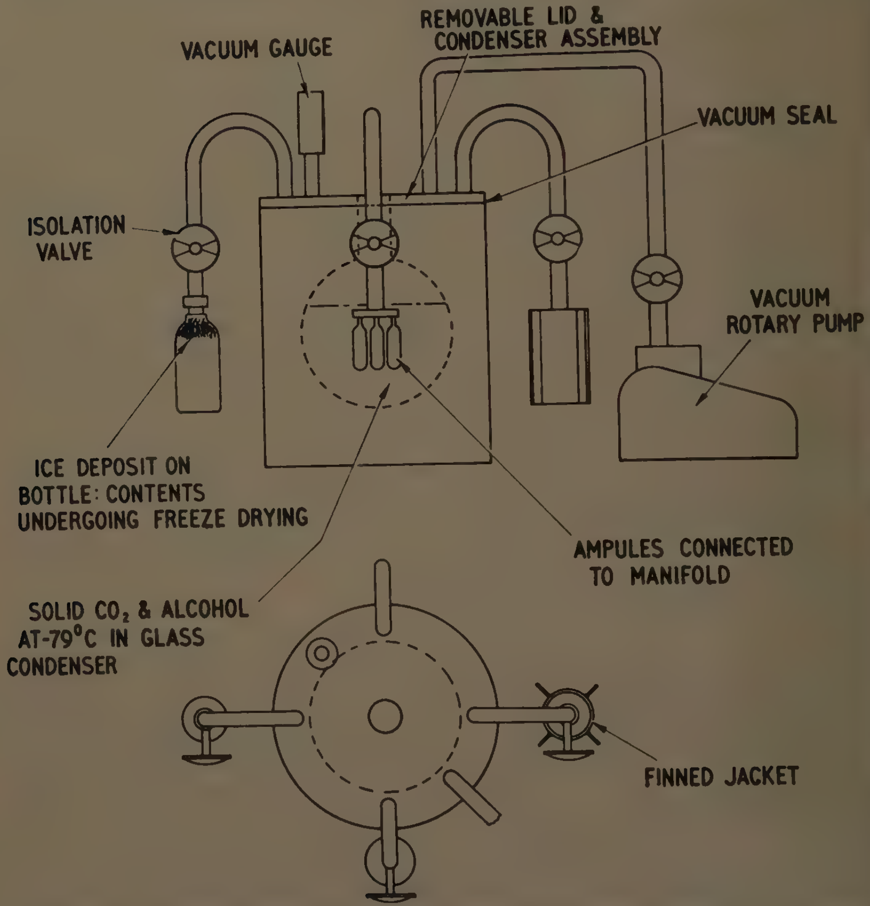
REMOVABLE LID & CONDENSER ASSEMBLY
VACUUM GAUGE
VACUUM SEAL
ISOLATION VALVE
VACUUM ROTARY PUMP
ICE DEPOSIT ON BOTTLE: CONTENTS UNDERGOING FREEZE DRYING
AMPULES CONNECTED TO MANIFOLD
SOLID CO₂ & ALCOHOL AT-79°C IN GLASS CONDENSER
FINNED JACKET

FIGURE 1. Schematic diagram of simplest equipment for laboratory-scale and pilot freeze-drying.

the final moisture content from the 6 to 7 per cent remaining after the ice has disappeared to a fraction of 1 per cent. The purpose of this further reduction in moisture content, which may, if carried out at room temperature, increase the total time by half, is to improve the shelf life of the material.

On completion of secondary drying the vacuum in the chamber is broken, preferably by admitting dry sterile air. Freeze-drying does not protect against oxidation and, if fats are present, an inert gas should be let into the chamber. After the removal of the trays the condenser is defrosted and drained, and the shelves are cooled in readiness for the next cycle of operation. The product may be transferred for secondary drying to another vacuum system of simpler design, using phosphorus pentoxide desiccant instead of a condenser, the system not being provided with a heating means. This second system, which may cost as little as one quarter of that of the primary drier, may be used when dealing with highly hygroscopic substances that cannot be heated above room temperature and therefore, unnecessarily, occupy the expensive primary drier for an extended period. The desiccant is renewed after the surface has become sticky, but before it has become completely liquid.

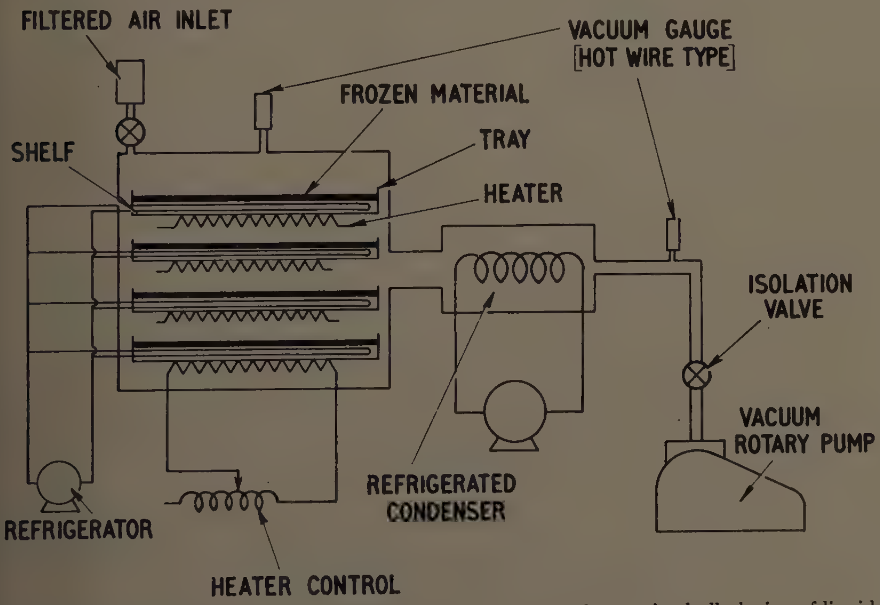

FIGURE 2. Schematic diagram of the simplest equipment for routine bulk drying of liquid solutions in trays.

The suspending medium. Greaves[9] has emphasized the importance of the part played by the menstruum in the success of freeze-drying. He suggests that the ideal medium consists of three components: a support material, a buffer for residual moisture, and a neutralizer of carbonyl groups. As an example, he quotes a solution containing 5 per cent glucose-free dextran, 5 to 10 per cent glucose, and 1 per cent sodium glutamate.

From the physical point of view the menstruum, together with the substance to be dried, should be homogeneous and should not have an unduly low eutectic, above −25° C. being convenient. It should form a coherent sponge when dry of about the same size and shape as the frozen mass, be of good texture and appearance, and be readily soluble, even in cold water, after drying.

Solutions containing a few per cent of sodium chloride, even when gelatin is present as a binding agent, are inclined to "crepitate," small crystals of dry salt breaking away from the mass and becoming entrained in the vapor stream. If they lodge against imperfectly protected metal surfaces, particularly cuprous or ferrous, they cause severe corrosion in the presence of moisture.

Acetic and lactic acids, although not frequently present, are among the corrosive agents most commonly encountered in freeze-drying. The latter has occurred in milk preparations when its presence, surprisingly, was not suspected. The former can attack even air-ballasted pumps, permanently spoiling their performance. The only adequate protection is a caustic trap.

The presence of as little as 1 per cent of alcohol, ether, or phenol used in an extraction process or for killing microorganisms may cause contamination of an unprotected oil-sealed vacuum pump. The same proportion of alcohol also can cause bulging of bulk material away from the surface of a containing tray, leading to doubling or trebling of the drying time, which otherwise can be achieved when good thermal contact is maintained.

When pharmaceutical preparations are being developed the possibility of their being freeze-dried can be considered at an early stage, with advantage, to prevent the need for their later adaptation for ease in drying. Where this has not been done, it has sometimes been necessary to freeze and dry at temperatures as low as −40° C. with a consequent increase in the cost of equipment and of the over-all drying cycle. In such a case it may be cheaper to retrace the later stages of development of the preparation.

The inclusion of a wetting agent[10] to promote dispersion during deep culturing is an aid to solubility of the dry product.

Very low concentrations of binding material, for example, 0.1 per cent of gelatin, do not yield a dry structure of sufficient bulk density, and the fine web of material resulting from drying may collapse or leave the container altogether.

Substances containing albumin, dextran, or sugar may exhibit a glazed surface shortly after drying begins. This may retard further drying seriously and lead to melting of the product. In such cases, if only incipient melting occurs, the product may continue to dry while shrinking slightly. The meniscus attached to the glass wall will then break away, and drying will occur through the side of the pellet. In vials of less than 20-mm. internal diameter the residual ice within the material will assume a conical shape. If the adhesive forces are greater than those of cohesion within the pellet, a starlike crack may develop in the material, radiating from the axis. In either case the dry pellet is unsightly, but the effects often may be overcome by treating the internal surface of the vial with a silicone preparation of the kind used in the food-bottling industry.

Containers. Where drying is carried out in a number of separate containers, glass has always been the preferred material because of the visibility it affords and its ease in sterilizing. Many operators use neutral glass, but in any case a fairly hard glass should always be chosen when rapid freezing is to be carried out because of possible breakage by thermal shock. Where materials, even when dry, are sensitive to light, the bottles may be brown or of some other color.

Vials for tray drying preferably should have flat bases of uniform thickness and, in this respect, mold-blown vials are superior to molded cast ones. A small bead of glass may remain in the base of a mold-blown container, and this may slightly reduce the thermal conductivity at that point. If a sufficient margin of time is not allowed for primary drying to be completed, it is sometimes found that a trace of ice remains in containers having this defect. The ice melts when the vacuum is broken, dissolving some adjacent material and causing a small cavity to appear in the base of the pellet.

Bottles of the transfusion type, if they are to contain more than 250 ml. of fluid for drying at the maximum rate, should have neck openings at least 20 mm. in diameter. Parallel-sided bottles are obviously to be preferred to the waisted type.

Unless special arrangements are made for holding them individually, vials should be of stable design so that they are not knocked over easily. If vials become tilted in a tray or on a shelf, their contents may not freeze in the usual time or they may not be dry at the end of a normal cycle.

Where vials are to be stoppered, particularly under vacuum, the flange should be flat and free from mold flash.

Ampules that can be sealed by fusing the glass are often preferred to stoppered vials, as a visual inspection will usually reveal failure of the seal, but objections have been made to the danger and difficulty in cutting open sealed ampules. Stressed ampules that break easily and cleanly at an indicated position without the use of a glass file reduce these hazards.

Ampules for centrifugal freeze-drying should also be mold-blown to the recommended tolerances of the freeze-dryer manufacturer, as otherwise the glass wall may be too thin to withstand centrifugal force, the neck of the ampule may snap, or the ampule may take up the wrong angle of inclination to the axis, resulting in a wedge of incorrect shape.

Methods of freezing. The number of methods employed for the freezing of fluids is legion, and only the main types are considered. In most cases an attempt is made to distribute the fluid in a thin layer to reduce the drying time. Often some attempt is made also to induce a high rate of freezing for the production of small ice crystals to give a uniform even texture to the material when dried, and improved solubility. This may conflict with the requirement for ease in drying if freezing is too rapid.

Shelf freezing is a self-explanatory title. It may be carried out in trays containing bulk material, in vials, or in flat-bottom ampules. Freezing may be done outside the drying chamber, inside the chamber on refrigerated shelves or, again, inside the chamber if this also contains the condenser, by the circulation of air by means of a fan. In the last case, freezing of a 1-cm. depth of liquid in vials to −25° C. may take several hours; otherwise, freezing takes from 1 to 3 hours. Rapid transfer of externally frozen material to the freeze-dryer is necessary to avoid partial melting before the vacuum is obtained. In this respect refrigerated shelves in the dryer are an advantage, particularly if the vacuum pump takes more than 5 min. to reach a pressure of 1 Torr.

Freezing is sometimes carried out in a separate cabinet even when the dryer has refrigerated shelves. This may be to reduce the "dead time" in the freeze dryer, as a shelf refrigerator does not permit rapid freezing of a large load. Time in the dryer may be saved if condenser defrosting and shelf freezing can take place simultaneously. Some operators freeze their containers on heavy metal blocks that act as a cold reservoir during transfer. However, these blocks can impose an extra load on the condenser at the beginning of the drying cycle if they have warmed slightly after removal from the freezing cabinet. Their excess heat is absorbed by the product as heat of sublimation. Transfer blocks are sometimes drilled with blind holes to take vials or ampules to improve the rate of freezing and as a further precaution against melting during transfer. Freezing then takes place toward the axis as well as upward through the base of the container, resulting in the formation of a "pip" in the center of the meniscus.

When freezing is carried out in the drying chamber itself and there is irregular contact between the container and the shelf, it is advisable, if the door is to be closed, to leave open the air admittance valve to the chamber to prevent a reduction in pressure due to condensation of atmospheric water vapor and a lowering of the temperature of the enclosed air. It has been found that even the slight drop in pressure that this engenders can increase the freezing time.

Shell freezing is suitable for containers of 100 ml. and upward. Such containers are usually rotated at 100 rpm about a horizontal axis and partially immersed in a refrigerated fluid that may be acetone-cooled to −60° C. or below with solid carbon dioxide. The rotation is stopped for a few seconds at intervals until a line of liquid freezes along the lowest part of the bottle. Freezing of the rest of the fluid as a uniform hollow shell then proceeds rapidly when rotation is resumed. The freezing time for 250 ml. of blood plasma in a 500-ml. transfusion bottle is about 10 min. As with vertical spin freezing, described below, the frozen containers are kept in a refrigerated store until a full drying load has accumulated. This permits liquid refrigerant to drain from the outside of the bottles so that the quantity that might be transferred inside a chamber dryer is kept small. A trace of alcohol seriously can increase the time taken for the vacuum pump to reach the correct operating pressure, and it is advisable to wipe bottles before loading the dryer. In vertical spin freezing, bottles are rotated at 750 to 1000 rpm about their vertical axes in a blast of cold air at −18° C. or in a refrigerated fluid that may be ethyl alcohol, 1:1 mixture of ethylene glycol and water, or methyl cellosolve at a temperature of −25° C. or lower.

The first method, devised by Greaves,[11] avoids the carry-over of liquid into the drying chamber. If the critical recommended temperature is maintained and the liquid has been freed of potential freezing nuclei by filtration, supercooling occurs, followed by a rapid snap freeze. Due to the comparatively high temperature employed, however, heat transfer is slow and the over-all freezing period for a 250-ml. load in a transfusion bottle is 1 to 1½ hours. It is not always possible to maintain the precise conditions of rapid air flow over the bottles and the critical temperature for a freeze yielding the small ice crystals that make blood plasma, when dried, uniformly pale. The contents of some containers may freeze with fernlike crystals that, when dried, appear darker than those just described, giving plasma a hemolyzed appearance. Rotation at 1,000 rpm produces some variation in shell thickness from top to bottom. This is marked when 400 ml. is spun in a 500-ml. bottle, the variation in thickness being 3 mm.

Rotation in a fluid gives a more rapid freeze than in air: 25 min. for 250 ml. and 40 min. for 400 ml. However, there are difficulties with the drive and in maintaining the necessary rotational speeds due to turbulence in the liquid.

With both methods it is desirable before freezing to store the fluid in the bottles for several hours at about 1° C. to reduce the shock when the glass is exposed suddenly to low temperature.

Centrifugal evaporative freezing. This process was invented by Greaves.[12] When the total pressure above a suspension or solution is reduced below its saturation vapor pressure, the fluid will boil, becoming cooled in the process. The saturation vapor pressure of water and ice at 0° C. is about 4.6 Torr and, if the ambient pressure is reduced below this value, an aqueous fluid will boil and freeze. Due to the evolution of dissolved air at a reduced pressure, frothing occurs, which can expel the fluid from the container. Also bubbles of froth, being thin, may dry from the liquid state, rendering protein insoluble, a process that freeze-drying is designed to obviate. Centrifugation at a speed so low as not to cause separation of most fine suspensions has been found to eliminate frothing during evaporative freezing. Single containers may be rotated about their own vertical axes, or a number of containers may be rotated about a common axis, to achieve this effect. In the latter case the containers usually fit into holes in a carrier and are inclined toward the axis at an angle of about 7° from the vertical to avoid their being flung out, or to prevent the loss of fluid through the neck. The forces needed to prevent frothing with this method are about 7 g. A typical carrier-plate assembly containing ampules in holes arranged in concentric rings from 10-cm. to 25-cm. diameter rotates at 800 rpm.

Breakage of ampules may occur if the angle of inclination is too great or if more than the recommended nominal capacity is exceeded. The wedge of fluid so formed then extends beyond the diametral plane of the ampule, and expansion of the ice on freezing ruptures the glass. Very dilute solutions or those containing gelatin tend to stick to the glass on freezing. In evaporative freezing the surface freezes first and the sheet of ice that forms, if it adheres to the glass at the edge, traps fluid behind it. When this expands on freezing it may break the glass. The effect may be eliminated by treating the interior of the ampule with a harmless silicone preparation.[13,14]

Due to the formation of a hollow cylindrical core of ice having high compressive strength, evaporative freezing of shells of liquid in vertically spun glass containers may also lead to breakage. Usually about 150 ml. of fluid is the maximum that can be frozen safely in this way in a transfusion bottle if no salts are present. Metal or plastic containers do not suffer from this disability, and 15 l. of fluid has been processed in a steel container by Record and Taylor,[14] who claim to have improved thermal contact of the product with the container by continuing to rotate it throughout the drying period. The addition of as little as 0.025 per cent sodium chloride produces a softer ice and overcomes the problem of expansion.

The advantage of evaporative freezing is that freezing and drying may be carried out in the same chamber; a common refrigerator is used for both. The wedges or shells formed by evaporative freezing have configurations favorable to short drying periods. Evaporative freezing of 0.5-ml. quantities in suitable ampules may take about 2 min.; freeze-drying, 4 to 5 hours. The time for 10-ml. quantities is about 6 min. for freezing and 20 hours for drying. A small quantity of water—15 to 20 per cent of the total—evaporates during freezing, but this has not been shown to cause harmful concentration of the fluid. In fact, fluids containing up to 40 per cent solids may be treated. When freezing is complete, the rotation is usually stopped.

The technique fails for fluids containing a high proportion of dryness buffer or substances such as dextran, which form a surface skin. It is also inapplicable where the thermal mass of the container is large compared with the heat content of the fluid, when an excessive quantity of water may evaporate to cool the container. Both difficulties may be overcome by the addition of water to the fluid, in the first case to reduce the concentration and, in the second, to increase the bulk.

Sterility. Relative rather than absolute sterility is often the aim in the various stages of pharmaceutical preparation, of which freeze-drying is one. It has been argued that chance contamination of a preparation to a level below the minimum infective dose is tolerable; also that contamination is most likely to occur before the drying begins, which will either render the contaminant inactive until water is added to the product or else destroy it altogether. In spite of this, precautions taken are becoming increasingly stringent, and the same high standards now are applied to veterinary preparations as to those for human consumption.

Opinions differ as to the desirable standards of sterility and as to how they can best be achieved. These are matters that I do not presume to judge, although I can describe some of the expedients adopted.

Where sterility of the product itself is to be maintained, it is obviously desirable to dispense it automatically into the containers and to load these mechanically in so-called "sterile" rooms supplied with filtered air at a pressure slightly above that prevailing locally. Few modern freeze-dryers can be used as autoclaves for sterilizing their own interiors. Washing out of the drying chamber with alcohol and fumigating the vacuum system with formalin or ethylene oxide do not harm most constructional materials, but it is necessary to protect the vacuum pump against contamination by the vapors of the antiseptic used. The rooms are fumigated or spray-disinfected when necessary, or irradiated with ultraviolet radiation either continuously or intermittently. The standard of sterility usually thought desirable and readily achieved is a count of 3 microorganisms per hour on a 10 cm.-diameter agar plate.

When, in the present stage of the art, human operators must be employed for some of the product handling, they take the usual precautions of wearing sterile clothing and masks. Protection from harmful exposure of the operators to radiation may be necessary. These well-known precautions are mentioned to emphasize the fact that it is possible to operate all or part of many types of freeze-dryer in a sterile area.

The ideal dryer is one in which the drying chamber only is accessible from a sterile working space, being sealed through a partition dividing the space from a machinery room containing the rest of the installation. The sterile room needs only to be large enough for the carrying out of such filling, prefreezing, and sealing operations as may be required in addition to the loading and unloading of the dryer. Maintenance is made easy by locating the working parts of the equipment outside the sterile zone. The instruments and controls may be situated within or outside the zone. I prefer the latter unless most of the controls are duplicated to allow direct monitoring of operation during servicing. In any case, whenever a single unit is divided, means of visual or oral communication between operators located by the various subunits should be provided.

A less ideal but still practicable expedient is to contain the entire installation in the sterile area. In such a case it may be necessary to protect the product from air-borne contamination. This usually occurs by downward settlement, and the provision of trays with close-fitting lids that are lifted or tilted to permit drying only after the chamber has been loaded and evacuated now is not considered necessary. Satisfactory protection can be given to bulk material by fitting trays with lids that remain in position throughout drying but, because of an annular vapor path around the edge, do not impede the flow. Also needlessly complex is the fitting of vials with fully seated stoppers that are raised automatically in the vacuum chamber in correct location for automatic insertion after drying. Several types of stopper are now available that remain in the vials during the drying process without interfering with the vapor flow.

When vacuum pumps are operated within a sterile area the exhaust should be ducted away.

A compromise sometimes may be reached by locating the pump or the refrigerator outside the sterile zone, with the remainder of the unit inside. Attention must be paid to the conductance of the vacuum-pumping lines and to the insulation of the refrigerant pipes if their length exceeds more than a few feet.

The presence of oil in bearings in a sterile area is esthetically objectionable, but probably not a serious hazard. While it may act as a trap for microorganisms, it is not likely to release them.

When it is not possible to operate in a sterile zone, it is usual to cover trays containing bulk material or vials with a bacteriological filter. Greaves[11] has found that gamgee, which consists of a controlled thickness of laminated cotton wool sandwiched between butter muslin or gauze, affords complete protection from air-borne bacteria without significantly restricting vapor flow. A nominal thickness of one-eighth inch is suitable, but greater thicknesses can be used. Individual bottles, for example, of the M.R.C. transfusion pattern, can be provided with separate disc covers of one-eighth inch gamgee held across the mouth of each bottle by a spring attached to the neck. It is usual to avoid stretching the material across the opening and to allow it to form a shallow dome in order to increase its effective breathing area. When insufficient binder is present in the suspending medium, filters can be blocked by migrating solid particles.

Filters of gamgee may also be used to close the top of a tray or drum containing ampules even when their contents are to be frozen evaporatively. The open ends of the ampules should not be allowed to come in contact with the filter material lest its effective breathing area be reduced. Where separate ampules must be covered for fear of cross-infection, each may be fitted with a cap of fluffy white lint.

Cotton wool plugs are not recommended during the primary drying stage, as it is impossible to control their density and hence their filtering and respiratory properties.

Gamgee releases fibers into the containers it protects which, although innocuous, can be objected to as they are visible in a clear suspension after reconstitution. I have found no treatment that will overcome this and yet retain the efficient filtering properties of the material.

Unless gamgee is employed, sterile air or filtered inert gas only should be used to break the vacuum at the end of the cycle. The air can come from the sterile zone itself, although additional filtering is desirable. Heat-sterilizable filter units of stainless steel packed with glass fiber are recommended. Fiber fragments are prevented from entering the diameter by a disk of sintered glass or stainless steel. Glass disks need support against the strain of the difference in atmospheric pressure to which they are exposed when the air admission valve is first opened.

Where operators must be protected from aerobic particles released from the substance being dried, gamgee filters are proabbly the only safeguard unless the operators wear cowls as well as masks. Here, again, careful selection of the menstruum may avoid difficulties.

Aerobic particles finding their way into the vacuum pump may be expelled in the exhaust covered with a film of oil. Protected in this way, they may survive under otherwise adverse conditions. Ceramic filters in the exhaust line reduce substantially, but not completely, the expulsion of oil mist from a pump that is pulling a good vacuum but is running on full air ballast. Paper or "absolute" filters may remove 90 per cent of all mist, but it is not known how long they will withstand the expulsion pressures. The exhaust may be bubbled through an antiseptic, but the risk of suckback when the pump is switched off should be kept in mind.

The measurement of vacuum. Vacuum in freeze-drying is expressed in terms of residual pressure. The international unit is now the Torr which, for all practical purposes, is equivalent to a pressure of 1 mm. of mercury. Similarly, the micron may be taken as 10^{-3} Torr or 10^{-3} mm. Hg.

It is clear that the pressure in the system varies from point to point, being highest close to the evaporating surface, lower near the surface of the condenser or the desiccant, and lowest at the entry to the pump. The ratio of the components of pressure also will vary throughout the system. These components are the partial pressures of water vapor and of air. Traces of solvents or antiseptics may be present, and also volatile flavors when foods are dried.

Attention should be paid to locating the pressure-sensing elements so that significant readings are obtained. If attached near an ice surface, a gauge may usefully act as a vapor-pressure thermometer.

Where routine freeze-drying is being carried out, it is seldom necessary accurately to measure the absolute pressure in the system as long as the readings are reproduced from cycle to cycle. In such a case a single discharge tube gauge, the length of the glowing column of which is a function of pressure and extinguishes at 10^{-2} Torr, may be sufficient.

Where equipment is being used for pilot or experimental work, greater accuracy is needed. The most generally suitable gauge for this purpose is the Pirani or hot-wire type, in which the temperature, and hence the resistance, of an electrically heated tungsten filament can be calibrated against pressure. The gauge has the advantages of measuring total pressure and giving continuous readings that can be recorded automatically. The earlier shortcomings, namely, that the main voltage supply needed frequent checking and that the total range covered was short, have been overcome in the Autovac type of gauge, in which the filament is maintained automatically at a constant temperature of about 200° C. and the range covered is from 70 Torr down to 1 μ. Filaments were formerly of platinum, which catalyzed the thermal decomposition of vapors and became contaminated. The tungsten filaments now used do not behave in this way; they are also less subject to loss of calibration when contaminated. Even blackened filaments are superior in this respect. Pirani gauges, although calibrated for dry air, may be recalibrated readily for water vapor, but will not indicate the relative proportions of air and vapor present.

Ionization gauges are not usually considered suitable for freeze-drying instrumentation, as they are prone to contamination and are not easily cleaned.

The McLeod gauge, although an absolute instrument against which all other types are calibrated, is not recommended for use in the presence of water vapor. The operation of the gauge depends upon the compression of a sample of known volume of a gas at low pressure into the closed end of a glass capillary, where its reduced volume and the increased pressure exerted by it can be interpreted in terms of its original pressure. Over the range of pressures prevailing in the drying process, condensation of water vapor during compression and adsorption on the glass wall affect the accuracy of the instrument. If a water vapor trap is interposed between the gauge and the system in an attempt to read only the partial pressure of air, air diffuses through the trap, eventually building up a pressure in the gauge equal to the total pressure in the system. Although this has been proposed as a method of reading total pressure, the time taken for equilibrium to be reached is long and indeterminate, and I do not recommend it.

The rotary vacuum pump. The purpose of the rotary pump is initially to evacuate air from the whole of the freeze-drying system, from atmospheric pressure to about 0.5 Torr, in whatever time is necessary to prevent prefrozen material from melting, and thereafter progressively to reduce the partial pressure of air in the system near the condenser to 0.01 Torr. If the material is on refrigerated shelves, initial speed in evacuation is not of prime importance. For the pump to fulfill the second part of its function, it must have sufficient residual speed to cope with leakage into the system and with evolution of gas.

Leakage, if considered most likely to occur at door seals, vacuum couplings, and constructional welds, is largely a function of the linear dimensions of the unit. If there is a chance of leakage through porous castings, then the probability of leakage of a given magnitude is a function of area. The size of the pump provided will increase as the volume of the unit, so that leakage, if it occurs, is likely to be more troublesome in smaller than in larger units. A manufacturer will probably test a system having a volume of 1 cu. m., at the rate of 10^{-2} lusecs for leakage. A lusec is a rate of leakage, under a pressure difference of 1 atmosphere, of 1 l./sec. at a pressure of 1 μ. Such leakage would

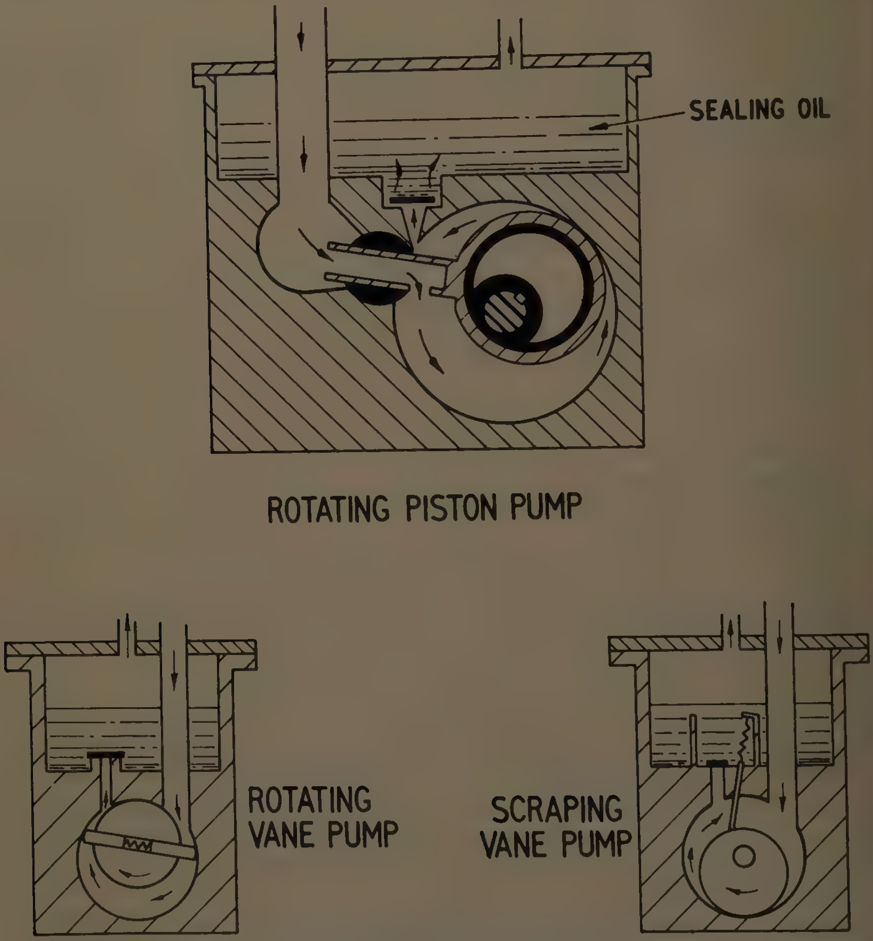

FIGURE 3. Schematic diagrams of the three principle varieties of the oil-sealed rotary pump.

be at least 100 times lower than the speed of the pump provided at the required operating pressure.

Frozen material examined in my laboratory has been found to have an air content of about 3 per cent of the volume of the frozen mass. Released over the period of primary drying, this could absorb at least one half of the speed of the pump at the prescribed pressure.

The oil-sealed rotary pump shown in FIGURE 3, of which three main varieties exist, is the type most frequently used in freeze-drying. In one variety of pump a cylindrical rotor is mounted eccentrically in a hollow cylindrical stator with which it makes contact at the top. Spring-loaded blades move in slots in the rotor, making contact with the curved wall of the stator and with end plates. A controlled oil feed lubricates the moving parts and seals the high- from the low-pressure or fine side. The mixture of vapors and gases to be pumped enters the inlet port, is trapped between the rotating blades, compressed, and expelled into the atmosphere. The design of the pump gives it a high-compression ratio, which means that permanent gases at pressures down to 10^{-3} Torr can be raised to pressures above atmospheric and expelled. Even lower ultimate pressures can be obtained by compounding 2 pumps in series or by incorporating 2 stages in a single pump. By this means the fine stage, connected to the vacuum system, does not work into the atmosphere, and air leakage from the rough to the fine side is reduced.

A limitation of oil-sealed rotary pumps in the past was their susceptibility to contamination of their sealing oil by condensable vapor; in freeze-drying, notably by that of water. Due to friction and work done in compression, a rotary pump may heat to about 70° C. during operation. At this temperature, the saturation vapor pressure of water is about 230 Torr—less than one third of an atmosphere. Any attempt to compress the vapor further results in condensation of water, which emulsifies with the oil. At first the pump may appear to remove water vapor from the vacuum system without loss of performance but, as the oil becomes increasingly contaminated, it finds its way to the fine side, when water is released and the vacuum is progressively spoiled. Prolonged operation under these conditions will cause the pump to rust or corrode unless it is made of stainless steel, which is unlikely.

Several methods exist to prevent contamination, including heating the pump above 100° C., recirculation of the pump oil through a stripper, and air ballasting. Heating of the pump leads to severe losses of oil through the exhaust, necessitating frequent "topping up." Moreover, this method is not effective against contamination due to the entry of liquid water into the pump.

In air-ballasting, a controlled quantity of filtered air is admitted to the pump when the rotating blade has isolated a charge to be pumped from the system, but before compression begins. Air and vapor are then compressed together to reach atmospheric pressure before the vapor components reach their own saturation pressures. For pumps running at 70° C. or above, operating on an air-ballast flow equal to about one tenth of their free air displacement, it is possible to pump water vapor entering at a partial pressure as high as 20 Torr. The vapors of solvents that are miscible with pump oil as, for example, acetone and ether, may also be pumped by this means without permanently contaminating the oil. The attack of corrosive vapors may be reduced by employing a suitable detergent oil containing an inhibitor.

Air-ballasting slightly affects the ultimate vacuum of a rotary pump so that, for freeze-drying, two-stage pumps are employed in which the second or rough stage only is ballasted.

The two chief features of interest in a rotary pump are its speed or displacement at different pressures and the ultimate vacuum it will obtain in a clean dry system (FIGURE 4). The time taken for a pump, unrestricted by pipelines, to reduce the pressure in a system free from leaks and contamination is given by

$$T = \frac{V}{S} \log_e \frac{P_1}{P_2}$$

where, in consistent units, V is the volume to be pumped and S is the average rate of displacement in the pressure range considered. P_1 and P_2 are the initial and final pressures.

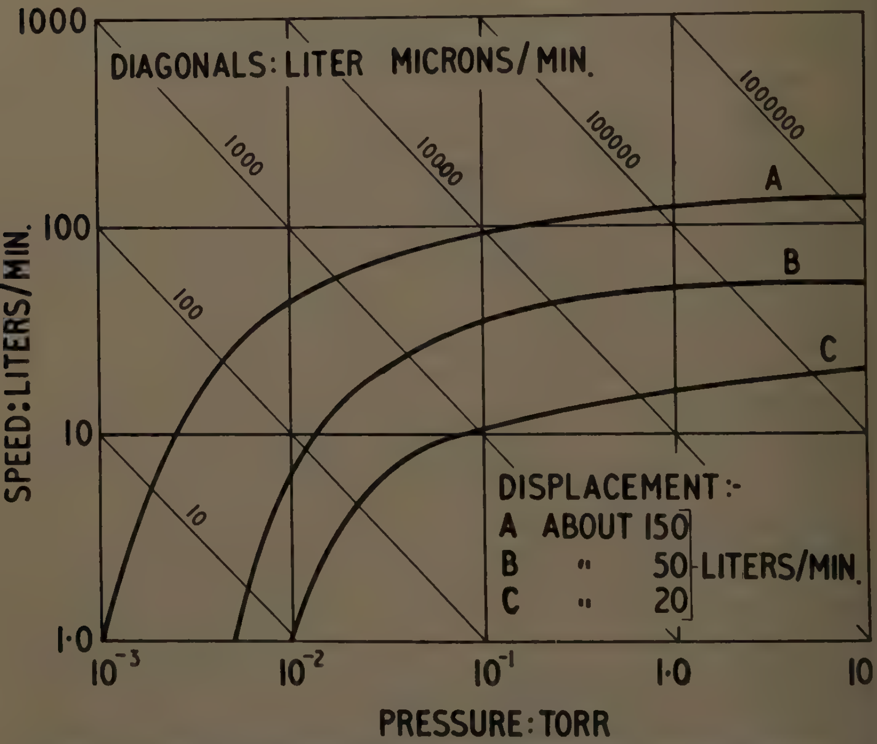

FIGURE 4. Speed or displacement at different pressures and the ultimate vacuum obtainable in a clean, dry system by 2-stage air-ballast pumps.

The volumetric efficiency of a pump falls off as its ultimate vacuum is approached. Pumps of the rotating-vane and scraping-vane types are not made with displacements much exceeding 1000 l./min. Above this value rotary piston pumps are used.

It is sometimes thought that, since modern pumps can deal with water vapor, they could be used in freeze-drying for direct vapor removal and eliminate the use of a desiccant or condenser. Unfortunately, the large specific volume of the vapor evolving in the freeze-drying process makes this impracticable. Assuming that the pump entry is situated immediately above the surface of the material being dried, the water vapor would enter the pump at a pressure of about 0.3 Torr. The volume of 1 gm. of water vapor at this pressure, corrected for temperature, is about 3000 l. A freeze-drying system holding 20 l. of frozen material to be dried in 14 hours would generate water vapor at a rate of 2 kg./hour or 6 million liters/hour. The system would have a volume of about 0.7 cu. m., which could be evacuated to a pressure of 0.3 Torr in 5 min. by a pump having a free air displacement of 1400 l./min. or 84,000 l./hour. Thus the pump would deal with only a small fraction of the total drying load.

Rotary pumps are provided with appropriate inlet nozzles. Lengths of connecting pipe, if free from sharp bends, of the same internal diameter as the pump inlet, and up to 1 m. in length, may be used in freeze-drying systems without loss of performance due to the restrictive effect of the pumping line.

Pumps having displacements above 500 l./min. frequently require water cooling.

It is advisable to pipe away the exhaust from a rotary pump, particularly if it is to operate on air ballast, which causes a continuous expulsion of oil mist. If the exhaust line has a fall toward the pump, it should be provided with a sump that can be drained, the sump being fitted as near to the pump as possible, to collect condensed vapors. The advice of the manufacturers should be sought in case the exhaust line imposes too high a back pressure.

A suitable 2-stage pump for equipment that dries 5 l. in 20 hours would have a free air displacement of 150 l./min. and an ultimate vacuum, when fully air ballasted on the second stage, of less than 0.01 Torr. Simple proportion applied to the rate of drying will give the appropriate pump for a unit of any capacity.

Diffusion pumps. So-called diffusion pumps have been advocated at various times by experimenters whose operations range from the drying of minute pieces of tissue to large-scale drying of blood plasma. I have found it possible, marginally, to reduce the drying time of bulk protein solutions by incorporating a diffusion type of pump in my equipment, but the greatest benefits are to be gained only when the condenser operates continuously at or below − 50° C., when the maximum thickness of the ice deposit on the coil does not much exceed 2 mm. and when the control of the product heating permits the supply of heat at the highest practicable rate for the greatest proportion of the primary drying period without danger of overheating the dry product.

A pump of this type always tends to strip a condenser but, in spite of its high displacement, the mass of vapor it can pump is small, and it is not an economic competitor of the refrigerated condenser. Its role in freeze-drying is to reduce partial air pressure and to assist the efficient operation of the condenser by removing the entrained air that accumulates around the coils.

If an advantage is to be gained, a pump of the same family but not a true diffusion pump, one known as a vapor booster, should be employed. A diffusion pump (FIGURES 5 and 6) operates on the principle of the entrainment of vapors and gases to be pumped by a high-speed jet of vapor of the pump-operating fluid, into which they diffuse from the system being evacuated. The fluid may be one of a variety of substances including mercury, although this is not suitable for use in freeze-drying.

The operating fluid is contained in a boiler at the base of the pump, the whole of which has been evacuated previously by a rotary pump to a pressure of 1 Torr or less. Vapor of the operating fluid from the heated boiler rises at high speed through a vertical tube, on leaving which it strikes a cone-shaped member and is deflected downward. The pump is connected at the top to the vacuum system, the pressure in which has been reduced previously by the rotary pump. Residual molecules of gas and vapor that diffuse into the pump from the system are carried downward by the vapor stream, and the returning vapor of the operating fluid condenses on the water or air-cooled wall of the body of the pump, the remaining air and vapors passing into the rotary pump. The vapor stream acts as a kinetic barrier against the back diffusion of the pumped gases into the vacuum system, and it will support small pressure differences, usually of a fraction of 1 Torr only.

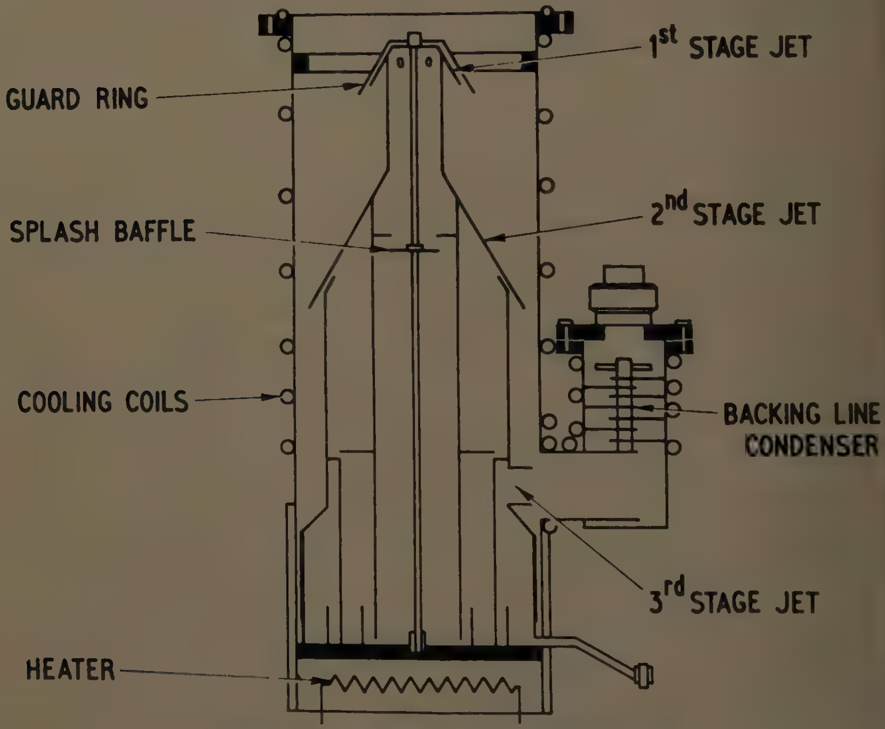

FIGURE 5. Schematic diagram of a diffusion pump.

A number of jet stages may be incorporated in a single pump. These stages, usually up to a maximum of five, increase the stability of the pump in operation and allow it to tolerate higher backing pressure.

Diffusion pumps have high volumetric displacements: one, 20 cm. in diameter, has a speed of 1000 l./sec. The ultimate vacua obtained by such pumps are of the order of 10^{-6} Torr.

Various oil-like fluids may be used in diffusion pumps provided they have a low vapor pressure at the temperature of the cooling water, thermal stability, and resistance to oxidation in case atmospheric air should be admitted accidentally while the pump is in operation. Of these, silicone fluids are recommended for freeze-drying. Glycerin, which is entirely nontoxic, can be used as a pump fluid, but it has a great affinity for water and should be kept under vacuum at all times.

Many modern pumps incorporate a simple fractionating system so that the low-boiling fractions resulting from decomposition of the fluid are distilled off and kept separate from the bulk of the operating fluid that reaches the upper jets.

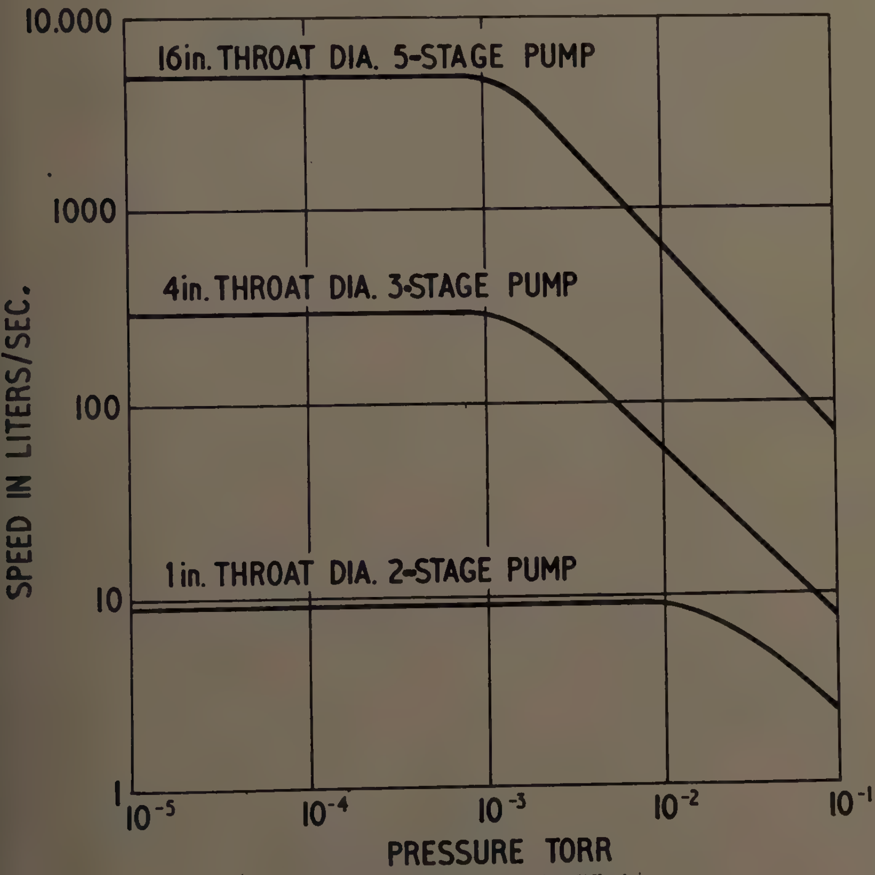

FIGURE 6. Speed (liters per second) of a diffusion pump.

A few of the molecules deflected by the jets have an upward component and tend to diffuse back into the vacuum system. It is advisable, therefore, to protect the system from this backstreaming by ensuring that the condenser is at all times interposed between the pump and the drying chamber. Precautions taken by manufacturers against backstreaming include the provision of a guard ring above the top jet.

Vapor-booster pump. The conventional diffusion pump has its highest speeds at pressures below 1 μ. A modification of the diffusion pump, the vapor booster

(FIGURE 7), is most efficient in the pressure spectrum from 1 Torr down to 1 μ which covers most adequately the freeze-drying range. At these higher pressures the pumped gases do not penetrate far into the vapor stream, but acquire momentum by collisions at the boundary. This pump can operate against a backing pressure up to 5 Torr, which gives it a considerable advantage over the true diffusion pump. At this pressure a rotary backing pump can deal with several times the quantity of vapor that it can handle at the lower backing pressure needed for diffusion pumps.

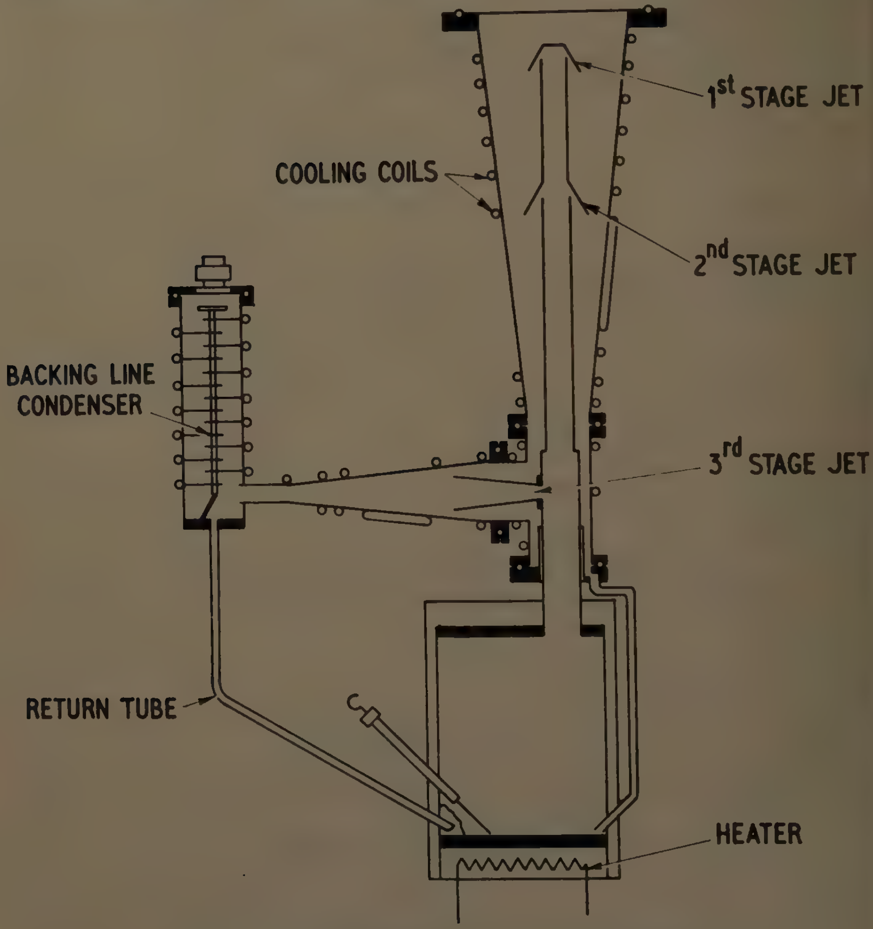

FIGURE 7. Schematic diagram of vapor-booster pump.

Performance curves for both types of pump are often plotted to show the variation of their mass rate of displacement with pressure (FIGURE 8). This information is of particular value in freeze-drying, where more vapor than air will be pumped. A characteristic of the vapor booster is its high mass displacement, which permits a comparatively small pump to maintain a safe low pressure against the evolution of vapor from the product and condenser if the refrigerator should fail. This is possible only if the product heaters are switched off and the drying and condenser chambers are well lagged. The rotary backing pump must be correctly sized to the booster, of course, or vapor will accumulate in the interstage connecting line and the critical backing pressure will be exceeded.

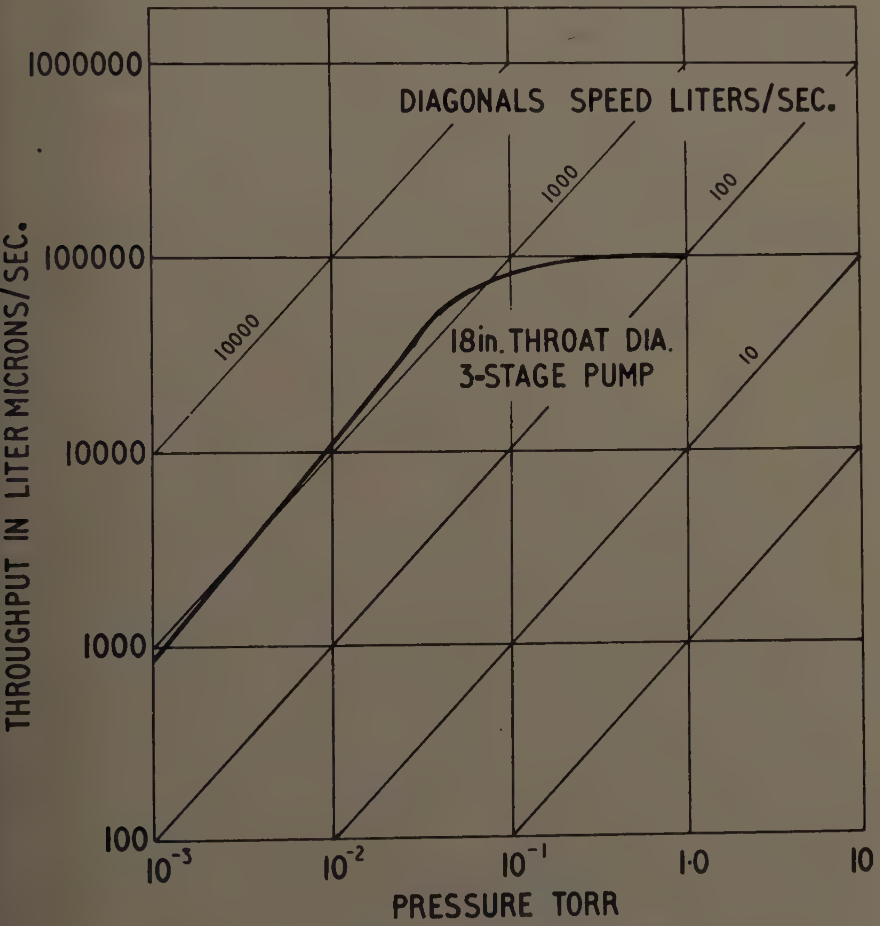

FIGURE 8. Throughput curves of vapor-booster pump.

Most pumps of this family have a warming-up period of 30 min. or more and should therefore be switched on in advance of being used. Although many operating fluids do not suffer from heating at atmospheric pressure, it is good practice to evacuate the pump while isolated from the system to below the critical backing pressure before switching on the heater. For the same reason the freeze-drying system should be roughly evacuated through a bypass line, with the diffusion pump isolated on the fine and on the backing sides.

The refrigerator. Refrigeration is among the most efficient means of pump-

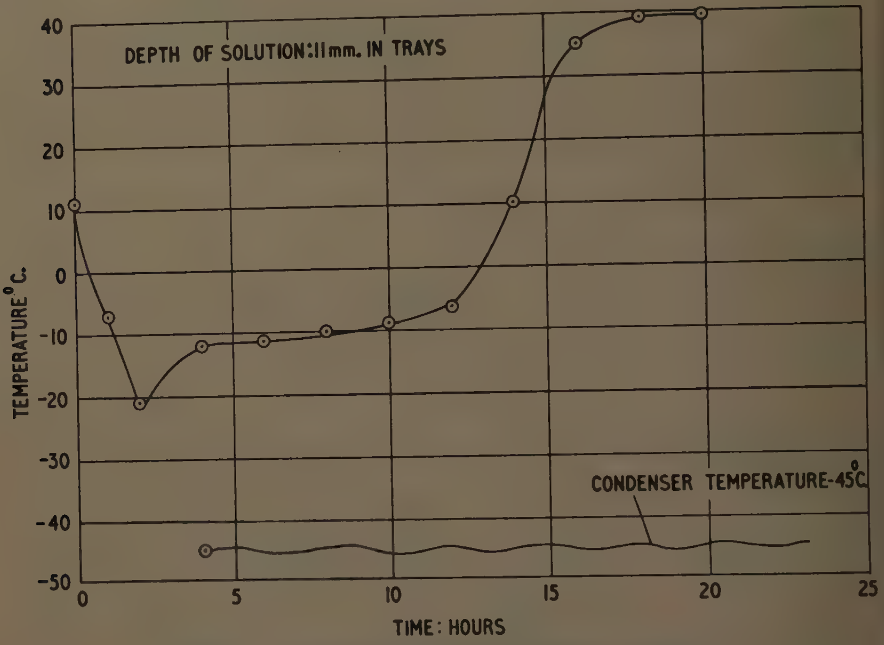

FIGURE 9. Freeze-drying curve for streptomycin sulfate.

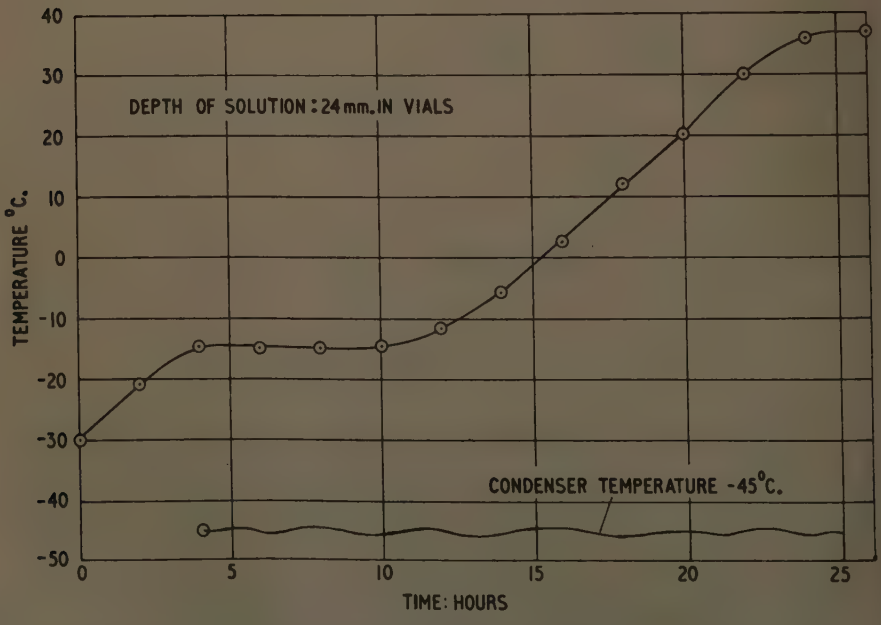

FIGURE 10. Freeze-drying curve for chloramphenicol succinate (Chloromycetin).

ing water vapor. As an understanding of either operation assists in clarifying the other, and as reliability is essential when refrigeration is used in freeze-drying, a description of the principles involved is included here.

An analogy has already been drawn between the freeze-drying process and a refrigerating system in which refrigerant fluid is circulated through pipelines by a compressor. If a restriction or throttle valve is introduced in the system, the compressor will cause a pressure to build up on one side of it and a partial vacuum to exist on the other. Refrigerant passing from the high- to the low-pressure region boils under the partial vacuum, taking heat from the pipeline, which may be in the form of an extended coil. Although this coil is known as the refrigerator evaporator, it is in fact the water vapor condenser of the freeze-dryer. The recirculated refrigerant vapor is cooled on the high-pressure side to liquefy in its own refrigerator condenser, from which heat is removed by air or water cooling.

With a low heat load on the refrigerator evaporator it is possible to increase the throttling by manual or automatic control, so that the flow of refrigerant is reduced, the suction pressure falls, and a lower boiling temperature is reached. If the duty on the coil increases, the throttle is opened to boost the flow of refrigerant. The constant-displacement compressor can furnish the higher mass displacement of refrigerant only by "pumping" at an increased pressure, which causes a rise in evaporator temperature.

As a refrigerator is a machine for transporting heat at a given rate from a lower to a higher temperature, its power consumption may be lower than the power absorbed at, or the rate of external heating of, the evaporator. However, the coefficient of performance of a refrigerator falls with evaporator temperature and, at −50° C., the power needed to drive the compressor is roughly equivalent to the power absorbed in the evaporator. This, in turn, is approximately equal to the rate of supply of latent heat of vaporization of the product provided artificially and by random heat gain. In the drying chamber allowance must also be made for the superheating of the vapor in its passage to the refrigerator, the lower sensible heat of the ice on the condenser than that in the product being dried, and the random heat gain by the condenser.

Temperatures below −50° C. may be obtained with single-stage compressors operating on Freon 22, but still lower temperatures require a 2-stage or cascade system with its attendant complexity. As the performance of a compressor falls rapidly with temperature, this is an argument in favor of operating at the highest safe temperature. Unless ammonia is used as the refrigerant, it is customary to employ thermostatic expansion valves for automatic metering of the flow. The size of orifice that such valves control and the degree of movement required are so small that sticking of the valve is the most frequent source of refrigerator failure. If the valve sticks in the open position or responds too slowly, it may pass more refrigerant than the load on the evaporator will vaporize. Incompressible liquid then will enter the compressor and damage the head valves. Sticking of the expansion valve in the closed position will starve the evaporator, and its temperature will rise. This can cause the product to melt.

Moisture in the refrigerating system forming ice in the valve orifice sometimes enters through the shaft seal on the compressor crankcase, which may be below atmospheric pressure. Vacuum dehydration of the refrigerator system is a remedy for the fault, and daily operation of the compressor to keep the shaft seal lubricated is imperative. When obtainable with the requisite performance, a hermetically sealed type of compressor driven from within is to be preferred to the so-called open type.

Condenser coils for trapping and freezing the water vapor that evolves in the freeze-drying process may be contained in the drying chamber itself, perhaps surrounding a stack of shelves or situated in an extension of the chamber below the stack. Alternatively, the coil may be in a separate vacuum chamber that communicates with the drying chamber by a pipe of high conductance. There appears to be no good technical reason for giving preference to any one of these arrangements. Adequate shielding of the shelves from the condenser, when all are in the same chamber, is necessary to ensure uniformity of heating of the shelves and to prevent the imposition of an unnecessary heat load on the condenser. Provided the connecting line between the two chambers has a low impedence to the flow of vapor, there may be a slight advantage in favor of the 2-chamber system in that the condenser may be isolated from the drying chamber for defrosting.

The condenser should baffle the pump effectively to prevent the direct pumping of evolved vapor. In an extreme case where this is not done the condenser might communicate with one end of the drying chamber and the rotary pump with the other. Residual air in the chamber and air evolving from the product would then be entrained by the vapor and carried away from the path to the rotary pump, finally to accumulate around the coils. Eventually a state of kinetic equilibrium would be established in which there was no further net transfer of air into the condenser chamber, but this would be reached only after an inhibiting blanket of air was formed around the coils.

With residual air pressures exceeding the recommended value ice tends to accumulate locally on the parts of the condenser nearest to the vapor source. Although the accumulation of ice may lead to a higher surface temperature, as further vapor is deposited, than the temperature at the more thinly coated surface, the effect may persist until adjacent turns of the coil are bridged, which may prevent the free escape of air into the pump, thereby aggravating the process. The effect is less serious when 2-stage air-ballasted rotary pumps are used, since these are normally capable of attaining the necessary low partial pressures of air while remaining free from contamination by water vapor. An unballasted pump may attain the necessary low pressures initially and, by so doing, create a situation favorable to the resublimation of some of the ice into it, causing contamination, slight loss of vacuum, and creating conditions favorable to uneven ice deposition.

The employment of a vapor booster pump adequately sized to the dryer completely eliminates the irregular building up of ice, thus permitting slightly higher rates of drying of a given product without fear of melting due to inefficient trapping by the condenser.

Condensers are normally operated at −50° C. although, for most substances drying at −25° C., a condenser temperature of −40° C. is sufficiently low. At this temperature the provision of a booster pump is scarcely worthwhile.

Condenser coils are normally designed with at least two minimum requirements: that when the freeze-dryer is fully loaded the coil surface area is sufficient to take the full load, when uniformly deposited without blockage, and that when drying of the product nears completion a maximum rate of vapor evolution does not cause a significant temperature rise at the surface of the ice already deposited on the coils.

Coils may be cooled either by direct expansion of a refrigerant as described above, or by circulation of a refrigerated brine. The former is nearly twice as efficient as the latter at a temperature of about −45° C. in terms of the horsepower of the compressor used and it therefore has much to recommend it. Advocates of the circulating method claim that it facilitates maintenance of a refrigerator, which then operates as an entirely independent unit, and that the brine tank acts as a cold reservoir in case of refrigerator failure.

In the case of direct expansion, multiple-pass systems are sometimes used to reduce the pressure drop of refrigerant vapor across the coil, causing lack of uniformity of temperature. When a separate expansion valve is used to control refrigerant flow through each separate coil, the chance of complete failure of a system due to the sticking of a valve is reduced. In a multiple-pass system it is possible to arrange the evaporators as a series of grids one behind the other. The grid farthest from the product is refrigerated first and, as the ice builds up, the anterior grid is refrigerated, this process being repeated in an attempt to ensure uniform deposition of ice.

Heating in freeze-drying. When drying is carried out on a manifold, heat is provided to the outside of the container partly by direct contact with the atmosphere and partly by condensation of atmospheric moisture, which usually freezes and forms an insulating layer. In the absence of any other source of heat the mass of ice deposited would equal that sublimed. In the case of shell-frozen material contained, for example, in Baxter flasks or transfusion bottles and connected to the freeze-dryer by negligibly restricting pipes, still air, even in a warm room, may not provide heat with sufficient rapidity for the product to dry at its maximum rate at the highest safe temperature. Drying can then be accelerated by directing a stream of air from a fan onto the bottles. Alternatively, the bottles may be fitted with jacket heaters. Equally effective, but simpler, are close-fitting finned copper tubes (FIGURE 1) that prevent the deposition of an insulating ice film on the containers and more effectively collect heat from the room.

In the case of materials with very low freezing points or preparations in containers of high thermal conductivity, where the room temperature is high or where there is much restriction to vapor flow in the connecting line, it may be necessary to insulate the container. Cotton wool may be used for this purpose, although jackets of expanded plastic or rubber are preferable, especially if the foam cells are not intercommunicating.

An ingenious method is employed by the human milk bank[15] of the Netherlands Red Cross, The Hague, the Netherlands. The manifold is enclosed in a plastic hood that can be fed with hot water, the vapor of which at a temperature between 25° C. and 55° C. condenses preferentially on the coldest parts of the bottle surfaces where heat is most needed.

In small glass freeze-dryers of simple design no special provision is made for the artificial supply of heat, although infrared lamps have been used for this purpose.

In a centrifugal freeze-dryer the wall of the cylindrical drying chamber is heated by a thermostatically controlled mat heater in contact with the outside and usually maintained at 40° C., which experience has shown to be a suitable temperature for most materials that can be frozen evaporatively. Although along the chamber radius the heat flux is not uniform, this effect has not been found to be harmful. The flux is concentrated at the axis of the chamber, but this is partly neutralized by the absorption of heat by successive concentric rings of ampules. Material in the outer rings dries first, then that at the center, followed by material in the intermediate rings.

Shelves may be heated electrically or by the circulation of a hot fluid. If they are also to be refrigerated, the suitably insulated heater mat is attached beneath the shelf; otherwise it may be placed on top. It is wise not to operate the heater much above 50 volts AC, to avoid the possibility of arcing at reduced pressure. If more than one shelf is provided, a heater of lower rating than the others should be fitted above the load on the topmost shelf. The maximum loading provided is about 1000 watts per square meter for bulk drying in trays and about three fifths of this value for drying in vials when the surface is less effectively used. Initial control is usually by autotransformer or energy regulator adjusted before the cycle begins. A single master thermostat or, preferably, independent thermostats on each shelf, are often fitted to prevent the temperature from rising above a predetermined safe value as the material approaches a state of dryness. With the values of shelf heating mentioned above, poor contact between a shelf and a tray can result in a temperature difference as high as 120° C. between shelf and product.

When imperfect contact is due to the fact that the shelf or the tray is not flat, the extent of the areas not in contact most probably depends on the greatest height of the gap between them. The heat traverses the gap partly by radiation and partly by conduction through the residual gas. It is my belief that the latter is the more significant of the two transfer processes, and that the heat flow across the gap for a given temperature difference is inversely proportional to the height of the gap and not independent of it. When the shelf or the platen are of light-gauge metal, heat is not readily conducted across the shelf and a local temperature difference is established that partly compensates for the lack of direct thermal contact.

If, however, material is being dried in vials contained in trays it is necessary to maintain a higher temperature differential between product and shelf due to the insulating effect of the glass. If, in addition, the shelf is fitted with a thermostat, and this is set at a safe maximum temperature for the product of, for example, 40° C. and if, furthermore, the position of the thermostat coincides with the gap, then it may begin to operate prematurely. The average heat input to the shelf therefore will be reduced, and the drying time of the product anywhere in the tray will be increased.

Where the shelf has a high thermal conductivity in a direction parallel to the surface, heat will be conducted away from local areas where poor thermal contact with the tray would otherwise cause "hot spots." If the rate of heating has been previously adjusted to a safe maximum for uniform contact, product contained in bulk in the tray may melt at any points where the tray and shelf are in good contact. If, on the other hand, material is contained in vials in the tray and if, as before, the heat input has been fixed for the maximum rate of drying, the thermostat will begin to operate whether it is near the gap or not. In this case the vials in that part of the tray that makes good contact with the shelf will dry in their usual time and the others will take longer.

It will be seen that an objection to direct electric heating of shelves is that, if they are thermostatically controlled, individually, they may tend to prolong the drying period by premature switching of the thermostat when thermal contact between the product and the shelf is poor. On the other hand, if the thermostat is not used and reliance is placed on manual control or on a time switch to reduce the rate of heating at the critical time, the high thermal mass of the shelves may cause harmful overheating of the product at the end of the drying cycle.

Fluid heating offers a solution of this problem. The fluid is usually one that has suitable physical properties over a temperature range from −35° C. to +80° C. and is also nontoxic and has a high flash point. The fluid is circulated from an electrically or steam-heated tank provided with a thermostatic control set at the value for the final product temperature. The shelves may contain cavities or cast-in serpentine pipes. When, as usual, several shelves are supplied from a single tank, the rates of fluid flow through all shelves should be equal. If the shelves exceed 1 square m., counterflow should be provided.

Initially the fluid should be cooled to the refrigerated shelf temperature, as its admission to a shelf, even at room temperature, at the beginning of drying may transfer an excess of heat to the product.

The tank heater is controlled as for mat heating but, when the product temperature begins to rise, the thermostat is brought into circuit. If the heater tank is unlagged, the temperature of the fluid and, hence, that of the shelves then falls rapidly to a safe value.

Electrically heated cylindrical containers that fit closely over bottles containing shell-frozen material may be made of light-gauge metal. They do not then suffer from the objection to heavy electrically heated shelves that areas making poor thermal contact do not increase in temperature. They also have a low thermal mass. When such heaters are arranged in honeycomb banks with the cylinders horizontal, radiation shielding must be provided to compensate for heat losses that otherwise give a drying time for bottles around the edge as much as 30 per cent longer than for bottles in the center of the array. As it is not practicable to precool a header of this type, it is necessary to load it rapidly with material prefrozen to a temperature well below its eutectic to avoid the risk of melting.

It is possible to freeze-dry shell-frozen material on or between fluid-heated shelves at the maximum rates possible by any other arrangement and yet to avoid overheating of the dry product. If the shelves can be refrigerated initially, there is no chance of melting.

By choosing a suitable wave length—one that causes energy to be absorbed preferentially by the ice and not by the dry material—infrared heating, it is stated, can be used in freeze-drying with great efficiency.[10] However, Greaves (*see* p. 682) has shown that the most efficient use of radiant heating, independent of wave length, is made when heating is from above onto an ice surface continually exposed to it and the vacuum by repeated scraping of dry material from the surface. Rates of drying approaching the theoretical maximum for pure ice can be achieved by this means. The method is suitable for bulk drying only. The outstanding problem to be solved appears to be the collection and removal from the vacuum system of the light powder formed by scraping, as this powder is at the mercy of the high-velocity vapor streams in the system.

Radio-frequency heating appears to offer an efficient means of generating heat within the ice, although care must necessarily be taken not to provide energy throughout the ice mass at a greater rate than it can be conducted to the surface and absorbed by sublimation without melting internally. Tests at about 1 mc. have failed due to the formation of glow discharges in the residual air and vapor. Tests at 1000 mc. have been more encouraging,[12] and claims have been made of the successful and economical freeze-drying of vegetables and fruit, including strawberries, by this means. Ideally, it should be possible to fill a vacuum chamber with radiation through a wave guide and to support the frozen product on shelves of some dielectric material. In practice, the lack of homogeneity of the product would probably necessitate its forming the dielectric between two parallel plate electrodes. Temperature measurement and control would present a problem.

Temperature measurement. An indication of the product temperature with an accuracy of 1 to 22° C. at all stages of freeze-drying is of greater significance than measurement of shelf or condenser temperature. The chief difficulty in achieving this objective is caused by the smallest practicable size of the available thermometer elements, whatever their type. The problem is particularly acute when 1-ml. or small quantities are being dried in vials, and where the frozen mass is initially less than 5 mm. in thickness.

As the important variables are the highest temperatures reached by the frozen mass and by the wholly dry material, a single thermometer element touching the bottom of a sample vial on each shelf is usually considered adequate. Elements in common use are thermistors, resistance thermometers, and thermocouples, this being their order of relative accuracy when used under normal operating conditions. However, the first two must be sheathed, and the over-all size of the elements, as a rule, is not less than 3 mm. in diameter and 1 cm. in length.

Thermocouple-measuring junctions need not be enclosed unless there is an objection, in the case of bulk material, that the bare wires may contaminate it. The junction and part, at least, of its leads should be of fine-gauge wire to avoid the conduction of heat along them, which could cause not only false readings but the premature drying of material in contact with them. To avoid setting up spurious potentials resulting in inaccurate readings, no junctions of intermediate metal should be introduced between the temperature-measuring and the reference junctions. Where the wires must be brought through the chamber wall, special glass seals through which hollow metal pins are fused may be used to carry them without a break. Thermojunctions, being easily made, are expendable, whereas thermistors and resistance thermometers are expensive. Of the last two, the interchangeability of the resistance thermometer makes it preferable to the thermistor.

Thermometer elements should be immersed in the fluid before freezing begins, and they must therefore be provided with wandering leads and plugs for rapid connection to the circuit. Plugs and sockets of special design should be used with thermocouples.

Care should be taken that conditions in vials or bottles used for temperature measurements do not differ from those in the other containers. Thick connecting wires should not be allowed to restrict the vapor flow through the necks of small vials or to interfere with the seating of stoppers or filters that are in position throughout the drying cycle. Where there is danger of this, a number of bottles are prepared with holes drilled in their shoulders through which the wires can enter. These bottles and their reconstituted contents may then be used repeatedly.

In a centrifugal freeze-dryer the temperature of the product during evaporative freezing may be measured only if a brush and slip-ring device can be fitted. Where this cannot be done, the temperature during drying after evaporative freezing may be measured if unconnected thermometer elements are introduced into selected ampules before freezing. When freezing is complete, the centrifuge is stopped, the vacuum is quickly broken, and the elements are rapidly connected before re-evacuation. This may be carried out with 5-ml. quantities of material; smaller amounts may melt before the vacuum is restored.

Sealing the containers. Much freeze-dried material is hygroscopic, and its container should be sealed to prevent reabsorption of moisture from the atmosphere.[8] If precautions are not taken, a dried sample weighing 1 gm. can reabsorb 2 per cent of its weight in moisture from the air in 1 min., although it may take several hours to reverse the process by drying under vacuum. When the substance is contained in bottles or vials, secondary drying is usually carried out in an evacuated enclosure. At the end of the drying period, dry sterile air or dry inert gas is admitted to the system, and the containers are rapidly removed for capping. Ordinary rubber stoppers are not suitable for this purpose unless sealed with wax, as they are permeable to moisture. This does not mean, however, that rubber is an unsuitable material for sealing vacuum systems that are continually pumped or in which moisture can be continuously trapped, as the small quantities of moisture that leak in are quickly removed.

Vials dried with suitably designed stoppers in position may have the stoppers pushed home while still under vacuum by means of a pneumatic ram. Butyl rubber is the most suitable material for the stoppers, as it has a permeability to moisture about ten times less than that of natural rubber. There remains the problem of evolution of moisture from the stopper itself. This can occur slowly over a long period and is not substantially affected by the presence of a dry gas if the vials are sealed at atmospheric pressure. It appears that exposure to the vacuum, which normally prevails during primary drying for 24 hours, is not sufficient to remove all the moisture present in small rubber stoppers used for closing vials of 5-ml. total capacity (private communication from P. N. Muggleton). The chance of serious rehydration of the dry product increases as the volume of the material is reduced. If stoppers of different sizes are assumed to have the same thickness, which is found in practice and is a compromise between mechanical strength and ease of puncturing by a hypodermic needle, then the permeability of the stopper or its moisture content decreases as the square of the diameter. On the other hand, the quantity of moisture needed to rehydrate the product from zero moisture content up to any given value depends upon the volume of the sample, which varies as the cube of its linear dimensions. Thus small quantities of material in small stoppered containers rehydrate more readily than larger quantities in appropriately larger containers.

The rate of diffusion of moisture through a stopper depends only on the partial pressure difference of water vapor acting across it and not on any gas- or air-pressure difference.

Ampules may be attached to a manifold for re-evacuation and flame sealing. Little skill is required for this operation if the ampule has been properly preconstricted with slight thicknening of the wall. The narrowness of the constriction required for safe vacuum sealing, without danger of cracking later, is such that the constriction is preferably made after primary drying, lest the product melt while drying is in progress.

A high-frequency, high-voltage spark generator may be used to induce a glow discharge in vacuum-sealed glass containers as an indication that the vacuum has been retained. The tester will also cause pinholes and cracks to show up in evacuated glassware.

Defrosting the condenser. Although in some freeze-dryers it is not necessary to remove the ice from the condenser after each cycle, a condition is eventually reached in which the condenser becomes inefficient due to the thickness of the ice deposit. If the spacing of the coils or pipes much exceeds 20 mm. this may occur before bridging takes place between adjacent coils. In some machines this bridging occurs before the ice deposit is otherwise thick enough to cause inefficiency. This may block some of the passages between the coils; the high rates of vapor flow along the unblocked passages then will result in inefficient trapping and accumulation of air above the coils, which may lead to melting of the product.

Methods of defrosting are germane to this paper, as they have some bearing on the economy and sterility of the process.

A condenser left to defrost naturally, with the refrigerator or refrigerant transfer pump switched off, may take several hours to lose all of its burden of ice. Where the drying process takes only an equivalent time, obviously the equipment cannot be used economically.

Where a coil is cooled by direct expansion, a process allows, in effect, for the running of the refrigerator in reverse, its evaporator and condenser being interchanged. A similar result may be obtained when the coil is cooled by a "brine" circulating system by heating the "brine." In both cases ice falls away from the condenser in large pieces that may block the drain unless it is provided with an opening at least 15 cm. in diameter.

More satisfactory is the circulation of heated air over the coils. The air should preferably be filtered or, at least, drawn from a sterile zone. The process is too lengthy if the coils are not uniformly coated, or if the maximum thickness of ice much exceeds 2 mm. Even so, with a 2-kw. heater it can take up to 40 min. to remove 10 l. of ice. The method can overheat the coils, which may be dangerous if they contain a refrigerant that exerts a high pressure at the temperature reached.

In my view the best medium for defrosting is water applied externally to the coils, although the efficiency is much higher if the condenser can be flooded rather than sprayed. The water may be at room temperature, although at least 7 l. are required for each liter of ice. A constant flow through a flooded condenser gives the most rapid defrost, particularly if the water is preheated to about 60 to 70° C. The maximum safe temperature depends on the design and finish of the condenser and the type of refrigerant in the coils. Flooding is feasible only if the condenser can be isolated from the vacuum chamber and rotary pump, or if the pipeline entries are above the highest point of the coil.

If the equipment is operated under sterile conditions the defrosting water also should be sterile. It may be conserved in a lagged tank provided with a heater and circulating pump. The tank should be sealed except for an overflow pipe for the surplus water derived from the melted ice.

Of the various mechanical means of defrosting that have been developed it is perhaps sufficient to mention only the rotary scraped type. In this the condensing surface is the interior of a hollow vertical cylinder, the wall of which is continuously scraped by rotating blades. Flakes of ice fall to the bottom of the condenser and can be removed intermittently through a vacuum lock.

The Freeze-Drying of Solids

Tissue drying. The necessary standardization of freeze-dried pharmaceutical preparations by bioassay may not have the highest statistical reliability, but the method does offer a means, if the number of samples taken is sufficiently large, of determining the degree of success achieved in the freeze-drying process of preserving the activity of the preparations. An attempt to judge the success of freeze-drying of human tissue for grafting by recording the case history of a patient after the operation presents far greater problems.

Heroic recording of all relevant information is carried out by Hyatt[18] in the tissue bank of the Naval Medical Research Institute, Bethesda, Md., but, despite the high degree of clinical success, it is not yet possible fairly to state whether a freeze-dried graft ideally should be vital or whether it should serve merely as a scaffolding for new tissue.

A discussion of this nature is not strictly proper to a paper concerned primarily with the physical and mechanical aspects of the subject. Nevertheless, the quality required in a good homograft will determine the conditions necessary for obtaining it, and there is not full agreement among operators as to what constitutes a satisfactory freeze-dried graft.[19, 20]

Hyatt stores harvested human tissue under aseptic conditions at −60° C. until required for drying. The samples are then transferred to a sterilized 18-cu. ft. drying chamber containing an upper drying and two lower condensing plates. The former can be heated or cooled by a circulating fluid; the latter are cooled by direct expansion to a constant temperature of −45° C. The refrigerating system can deal with a 10-l. load in 24 hours.

The system is evacuated by an oil-sealed rotary pump in 30 min. to a pressure of 5 to 10 μ, as indicated by a McLeod gauge provided with a chemical vapor trap. The shelf temperature rises to 0° C. within 24 hours and to 30° C. in 9 days. The prolonged drying period is to ensure a low residual moisture content in bone that is dried together with soft tissues. In spite of the length of the drying period, it seems possible that these materials do not undergo true freeze-drying. Due to their structure, moisture is slow in escaping, and the rate of sublimation may not be sufficient either to ensure that all free moisture has been removed after 24 hours or that the remaining moisture can escape at a sufficiently high rate thereafter to maintain the frozen state.

When tissue preservation is required[21] for microscopic examination or histochemical study, it is necessary to select only specimens having a maximum thickness of about 1 mm. in one direction for rapid freezing immediately after removal from the living donor. Freezing is accomplished by plunging the tissue in *iso*-pentane cooled to about −150° C. by liquid nitrogen or into liquid propane at about −185° C. These and other intermediate cooling liquids avoid the relative delay in freezing caused by the evolution of thermally insulating bubbles around a specimen dropped directly into liquid nitrogen.

Drying is carried out at a temperature between −30° and −40° C. in an evacuated enclosure maintained at a temperature no more than 1° or 2° C. above the desired temperature in the tissue.

Gersh and Stephenson have dealt with the subject exhaustively in a classic contribution.[24] They conclude that a partial pressure of air of 10^{-4} Torr is sufficiently low for maximum rates of drying regardless of temperature and that 10^{-3} Torr is desirable for drying at −40° C. They regard these pressures as attainable by a rotary-vacuum pump. They also state that a trap cooled by a solid carbon dioxide-alcohol mixture at −78° C. or containing phosphorus pentoxide at room temperature, if of reasonable size, is suitable.

From a graph in their paper the computed values of minimum drying time of guinea pig liver are given as 1 hour at −30° C. and in excess of 1 day at −60° C. for slices of which one half the least thickness is 0.3 mm. These authors have no particular preference for equipment of special design and are satisfied with drying in a refrigerated test tube.

Rey[21] proposes attaching the specimen to a comparatively massive block of metal for control and observation of tissue temperature.

I wish to add that, if any virtue is to be claimed for apparatus especially designed for tissue drying, it should be of simplicity consistent with reliable operation over the period of drying with the minimum of attention.

The Freeze-Drying of Foodstuffs

When drying foodstuffs the chief problem is to make the process economically attractive. Capital and running costs for freeze-drying pharmaceuticals, although high, are usually negligible in terms of the monetary or rarity value of the substances treated.

The scaling-up of the freeze-drying process to deal with ton quantities in a single batch goes part of the way in reducing the installation cost, since the price of equipment does not rise in proportion to its output. A big reduction in the cost can be brought about, however, only if the average rate of drying of about 0.5-mm. thickness per hour is drastically reduced.

The heated-plate contact process developed for conventional vacuum drying[22] shows the greatest promise. The drying chamber contains a stack of shelves that are cavity-heated by water, the direction of flow of which can be reversed at intervals to ensure uniformity of heating. Prefrozen sliced foodstuffs, either raw, blanched, or precooked, of roughly uniform thickness, are placed on the shelves, which are movable and linked in such a way that they can make contact with the material above and below at a controlled pressure. Difficulties involving the sticking to the plates of the material being dried have been overcome by the use of an expanded metal sheet that is placed above and below each shelfload. Compression exerted by the plates forces the metal into good thermal contact with the material while permitting the free escape of vapor, the restriction of which leads to melting and sticking of the material. Slight shrinkage occurs as drying proceeds, and the plates are advanced to overcome this.

The system is evacuated initially by water ejectors and then by 2-stage steam ejectors.

The material is usually prefrozen in an air blast, but minced meat may be frozen evaporatively in the chamber. Evaporative freezing of whole pieces of meat leads to the formation of an only partially permeable skin. The effect can be overcome partly by wetting the surface of the material before freezing.

Cod steaks 15-mm. thick, originally dried in about 11 hours by the standard contact-plate process, can now be dried in less than 7 hours.

An ingenious arrangement has been employed by Smithies,[23] who has impaled meat on spikes that conduct heat into the frozen mass and form passages for the escape of vapor. Opposing spikes are attached to metal plates that can, however, be heated directly or by radiation. The method permits extremely short drying times, but the spiked plates are expensive to make and the bulk loading of the drying chamber is less than with the contact-plate method.

Matters for Investigation

In spite of recent intensive study of the mechanism of freezing that has made possible the frozen storage of substances that had previously defied this treatment, the recent developments in freeze-drying have been less spectacular. In my opinion the following matters are in urgent need of investigation.

While methods of freeze-drying by the flow of dry gases have yielded successful results in various hands, no attempt has yet been made to compare this technique in terms of ease and economy with conventional vacuum freeze-drying.

It is known that different desirable properties in freeze-dried material are influenced by the rate of freezing. It is also known how they are affected by concentration of the salt and other constituents before freezing. Again, salt concentration and rates of freezing have been shown to condition the crystal structure of frozen material, thereby influencing the practicable rates of freeze-drying. Insofar as is known, no attempt has yet been made to correlate these results.

Many substances do not survive freezing and thawing, although of these some survive freezing, freeze-drying, and rehydration. Analogies may be drawn between freezing and dehydration and between thawing and rehydration. No work has been done to compare rehydration and thawing.

The use of glycerol as a protecting agent in freezing greatly increases the potentialities for frozen storage of viable substances. Unfortunately, glycerol has unsuitable physical properties for freeze-drying. A substitute for glycerol is required so that freeze-drying can be carried out after freezing.

Greaves' method of centrifugal evaporative freezing, although known to fail in certain conditions, can yield higher titers of viable microorganisms when the product dried is reconstituted than after other methods of freezing. It is understood that Greaves is investigating this at present.

No systematic attempt has been made to study the effect of residual gas pressure in a freeze-drying system.

No critical comparison has been made of methods of heating during freeze-drying—these include conductive, convective, radiant, and dielectric heating.

Attempts have been made to examine the economics of the pumping of vapor in freeze-drying, and steam ejectors have been compared with combinations of pumps and condensers. Methods of high-speed mechanical pumping, possibly in conjunction with water-sealed pumps or water-cooled condensers, might now provide simpler and cheaper alternatives to the methods of pumping and trapping at present in use.

Work on improving the economics of the freeze-drying of foodstuffs needs intensification and evaluation on a scale comparable with other methods of food processing, both batch and continuous.

Contrary to earlier beliefs that moisture contents below 1 per cent were necessary for the stability of a freeze-dried product, it is now known that viable substances can be overdried, with loss of titer immediately after drying and with further loss during storage. Additional work can profitably be done on the role played by the adjuvant at present employed to prevent overdrying.

The next logical step in the design of freeze-drying equipment will be toward complete automation and continuous processing. This is obviously a problem for the equipment manufacturers.

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DISCUSSION

JOHN L. STEPHENSON: I have a brief comment on the relation between vapor flow and heat supply. We always have the general relation

$$dQ/dt = L\,dm/dt \tag{1}$$

where dQ/dt is the net rate at which heat is supplied to unit area of the interface between dry shell and frozen interior, L is the latent heat of sublimation per gram of ice (numerically assumed to be 700 cal./gm. in our following computations), and dm/dt is the rate of sublimation. We also always have the relation (if back flow of vapor from the cold trap is assumed to be negligible)

$$\frac{dm}{dt} = \frac{\alpha f P_s}{(2\pi R T_s)^{1/2}} \tag{2}$$

where T_s is the temperature of the interface, $\alpha P_s/(2\pi R T_s)^{1/2}$ is the maximum rate of sublimation of pure ice at temperature T_s—α being the accommodation coefficient, P_s the saturated vapor pressure, R the gas constant per gram, and f a dimensionless factor less than unity that gives the probability that a water molecule leaving the interface escapes from the system. Measurements on a variety of systems[1-3] have shown f to be of the order of 10^{-2} to 10^{-4}. (In general,

f decreases as the thickness of the dry shell increases. For reasons not yet fully understood it appears to be more nearly inversely proportional to the square root of the thickness of the shell rather than the first power.) The interface temperature will adjust itself until the equation

$$\frac{dQ}{dt} = L\frac{\alpha f P_s}{(2\pi R T_s)^{1/2}} \tag{3}$$

is satisfied. For precise computations one would use vapor-pressure tables and all available information on heat transfer in the system. However, some useful approximate computations can be made. From the Clausius-Clapeyron equation

$$\ln\frac{P_1}{P_2} = \frac{L}{R}\left[\frac{1}{T_1} - \frac{1}{T_2}\right] \tag{4}$$

or directly from vapor pressure tables for ice, the factor $P_s/T_s^{1/2}$ can be shown to decrease by very nearly 10 for every 20° C. decrease in temperature T_s. For $-50°$ C. (more precisely $-53°$ C.) the value of $L\alpha P_s/(2\pi R T_s)^{1/2}$ is approximately 1 w/cm². Thus we obtain the approximate relation

$$\log_{10}\frac{dQ}{dt} = \log_{10} f + \frac{t_s + 50}{20} \tag{5}$$

where dQ/dt is in w/cm.² and t_s in degrees Centigrade. If the supply of heat is constant or a known function of interface temperature, the interface temperature can be computed from EQUATION 5. If dQ/dt is nearly independent of interface temperature, that is, nearly constant, it is possible to obtain a simple expression for the difference between the interface temperature of the actual specimen and a piece of pure ice in the same drying situation. Since for ice $f = 1$, $\log_{10} f = 0$, and for both specimen and ice $\log_{10} dQ/dt$ is the same, we find from EQUATION 5

TABLE 1

r	$r + \log r$	r	$r + \log r$
9	9.95	0.9	0.854
8	8.90	0.8	0.703
7	7.85	0.7	0.545
6	6.78	0.6	0.378
5	5.70	0.5	0.199
4	4.60	0.4	0.002
3	3.48	0.3	−0.223
2	2.30	0.2	−0.499
1	1.00	0.1	−0.900

$$\Delta t_s = 20 \log_{10} f \tag{6}$$

EQUATION 6 predicts that with the same heat supply biological specimens will dry at temperatures 20 to 60° C. above pure ice. Experimentally, differences this great are usually not observed, because the supply of heat usually decreases somewhat with increase in interface temperature. In many situations to a good approximation[1] we have

$$dQ/dt = K(t_e - t_s) \tag{7}$$

where t_e is the equilibrium temperature that the specimen would achieve in the absence of any sublimation cooling, and K is a general coefficient of heat transfer from the external environment to the specimen. If we eliminate t_s between EQUATIONS 5 and 7, we obtain

$$\log_{10} dQ/dt + (dQ/dt)/20K = \log_{10} f + (t_e + 50)/20 \tag{8}$$

where dQ/dt is in w/cm.² and K in w/cm.² °C. and t_e in °C. If we replace dQ/dt by $L\,dm/dt$ we obtain an analogous relation for mass flow.[1] In general, given K, f, and t_e, EQUATION 8 must be solved by trial and error for dQ/dt. This process can be simplified greatly by writing EQUATION 8 in the form

$$\log r + r = \beta \tag{9}$$

where $r = [dQ/dt]/20K$, and $\beta = [20 \log f - 20 \log 20K + t_e + 50]/20$; r might be called a "reduced" heat flow for the system and β a "reduced" temperature. For a given value of β, EQUATION 9 has a unique solution. Once these are computed or tabulated, analysis of any system described by the basic EQUATIONS 5 and 7 is simple. For $r > 10$, we have $r \cong \beta$; and for $r < 0.1$, $\log r = \beta$. Thus, only in the region $10 > r > 0.1$, corresponding to $10 > \beta > -1$, are tabulated or plotted values of the function $r + \log r$ needed. Some tabulated values for this range are given in TABLE 1. The use of these will be illustrated by an example. Suppose $f = 10^{-2}$, $K = 0.04$ w/cm.² deg, $t_e = 25°$ C. (The values of K and t_e correspond to radiative transfer of heat to a well-insulated specimen in a vacuum chamber, the walls of which are at room temperature.) The value of β computed from these values is 1.85. Therefore, we seek a solution of the equation

$$r + \log_{10} r = 1.85 \tag{10}$$

Interpolating from the table, we find $r = 1.6$, or by a quick slide-rule computation, $r = 1.65$. Since $20\ K = 0.80$, we obtain immediately $dQ/dt = 1.32$ w/cm.², $dm/dt = 4.4 \times 10^{-4}$ gm./sec. cm.²; and from EQUATION 7, $t_e - t_s = 33°$ C., $t_s = -8°$ C. In contrast, for pure ice, where $f = 1$, we find: $\beta = 3.85$, $r = 3.3$, $dQ/dt = 2.66$ w/cm.², $t_e - t_s = 67°$ C.; $t_s = -42°$ C.

In summary, this computational scheme greatly simplifies the approximate analysis of certain drying problems, but possibly its greatest utility is in showing how the vapor conductivity and heat conductivity affect the heat flow.

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THE APPLICATION OF HEAT TO FREEZE-DRYING SYSTEMS

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The speed of drying in any freeze-drying system depends directly upon the rate of application of the heat that is necessary to supply the latent heat of vaporization of the ice. The moment an attempt is made to increase the rate of heat application, problems begin to mount rapidly.

If the layer of material we are attempting to dry were infintely thin, the amount of heat that could be applied would be infinitely great, and the speed of drying infinitely fast. However, the moment we become practical and give the material thickness, a number of complications are introduced.

FIGURE 1 depicts schematically a block of frozen material of thickness *AB* standing on a heater plate *A*. Let us assume that the material contains a eutectic mixture at −18° C., so that it is not safe to raise any of the block above −20° C. during drying. Having set the heat input so that the heater plate is at −20° C., we find a temperature gradient through the block to −35° C. at the drying surface. The gradient between the surface at −35° C. and the condenser at −40° C. is caused by the obstruction to the flow of water vapor to the condenser induced by the actual form of the apparatus, so that in design every effort should be made to keep this obstruction minimal by allowing a large passage for the flow of vapor, keeping this passage as short as possible and keeping the surface of the condenser as large as possible.

As drying proceeds, the drying surface will recede to point *B′*, and the temperature will be found to have risen to −30° C. This rise in temperature is due to the fact that the dried material is now offering further obstruction to the flow of vapor to the condenser. The degree of this obstruction will vary with the type of material we are drying and with its concentration. It may well be that the temperature at *A* begins to rise above −20° C.; consequently, it may be necessary to reduce the heat input and hence the speed of drying as it proceeds. Conversely, with less dense materials, the temperature at *A* may fall so that the heat input may be increased, and drying may proceed more rapidly.

A further deduction can be made from this diagram, namely, that the thicker the block of material to be dried, the less heat it is possible to apply without danger of causing thawing at the point of contact with the heater.

Because of these difficulties, in any practical freeze-drying method every effort is made to increase the surface and decrease the depth of the material being dried, and many ingenious devices such as spin-freezing around the inside periphery of bottles, have been utilized.

This basic, fundamental problem is the cause of the inefficiency of the method, and it is not overcome by using radiant infrared or radio-frequency heating.

If, instead of heating by conduction or radiation from below, the heat were applied by radiation from above, a very different set of conditions would result. There would be no danger of causing thawing in the lower layers of the material; the danger now would be that of raising the dried material to an unsafe high level.

A practical experiment along these lines, drying 20 per cent coffee extract with a eutectic of −20° C., and a depth of 1½ in., showed a fourfold improvement in the rate of drying without any detriment to the flavor or solubility of the dried product.

If there were no danger of overheating the dried material at the surface it should be possible to increase the rate of heat input and, consequently, the rate of drying very considerably.

To test these theories experimentally, a 2-l. volume of distilled water was placed in a rectangular pan of area 44 sq. in. and was frozen. Three thermocouples were also frozen into the ice, one just below the surface, a second as near as possible in the center of the block, and the third at the bottom which was 3 in. below the surface. A heat input of 100 w was then beamed onto the surface. As each thermocouple came nearer the drying surface, its temperature dropped to −36° C. and, once freed of ice, rose rapidly out of the range of the recording apparatus. Drying of the 3-in. depth was completed at a temperature of −36° C. in 20 hours (FIGURE 2). This drying rate could have been considerably increased had the refrigerator been capable of neutralizing more heat. This experiment, however, was adequate to demonstrate the correctness of this theoretical approach and to justify the next step, namely, to try to apply this method to materials with a solid residue. It was required only to devise a method for the continuous removal of the dried material from

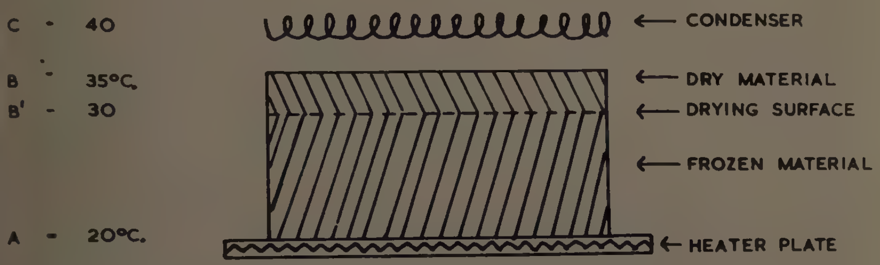

FIGURE 1. Diagrammatic representation of temperature distribution in a freeze-drying system.

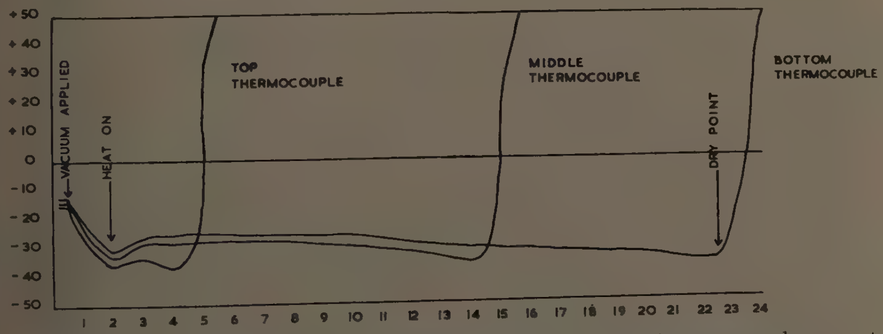

FIGURE 2. Drying curves for 2 l. of distilled water ice prefrozen in a rectangular pan at a depth of 3 in. Heat input, 100 w, vacuum, 0.05 mm. Hg; condenser temperature, −50° C.

the surface, which theoretically should be easy, since the friable dry material should readily separate from the solid, hard-frozen material beneath it. If this proved possible, the drying of great thicknesses of material at very high rates should also be possible.

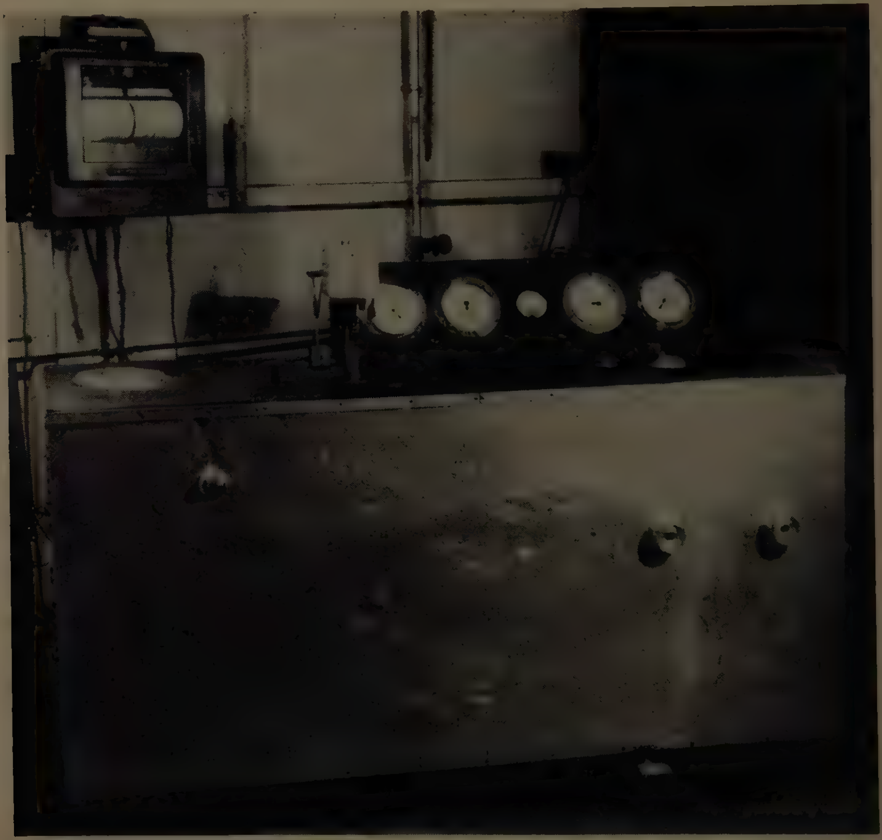

FIGURE 3. Freeze-drying apparatus used in these experiments. See text for description.

Apparatus

The experiments were carried out in an existing freeze-drying apparatus (FIGURE 3). In this apparatus the drying chamber is made from a steel tube 16 in. in diameter. The ice condenser occupies the bottom portion of the drying chamber and is refrigerated by direct expansion of Freon 22 by a one-third horsepower sealed Prestcold Compressor. The condenser of this refrigerator is placed in an alcohol tank refrigerated to −25° C. by a second compressor operating on Freon 12. The cold alcohol in the tank can be circulated through a tray in the drying chamber placed above the ice condenser coils and also through a small bath in which ampules of material may be prefrozen by vertical spinning. A number of thermocouple circuits are available in the drying chamber, and these are connected to a multipoint recording galvanometer for the continuous recording of temperatures.

The available space for the experimental apparatus in this plant was a cylinder 11 in. deep and 11½ in. in diameter.

Of the many forms that an apparatus for the continuous scraping of the surface could have taken, limited by these particular space considerations, the form shown in FIGURE 4 was adopted. This consists of a brass plate 9½ in. in diameter, driven by 2 synchronous clock motors through reduction gears so that the plate makes 6¾ rpm. Two motors were used, since the power developed by one was inadequate. The material to be dried is cast in the form of a halo in a brass mold, the center and side of which are removed before drying begins.

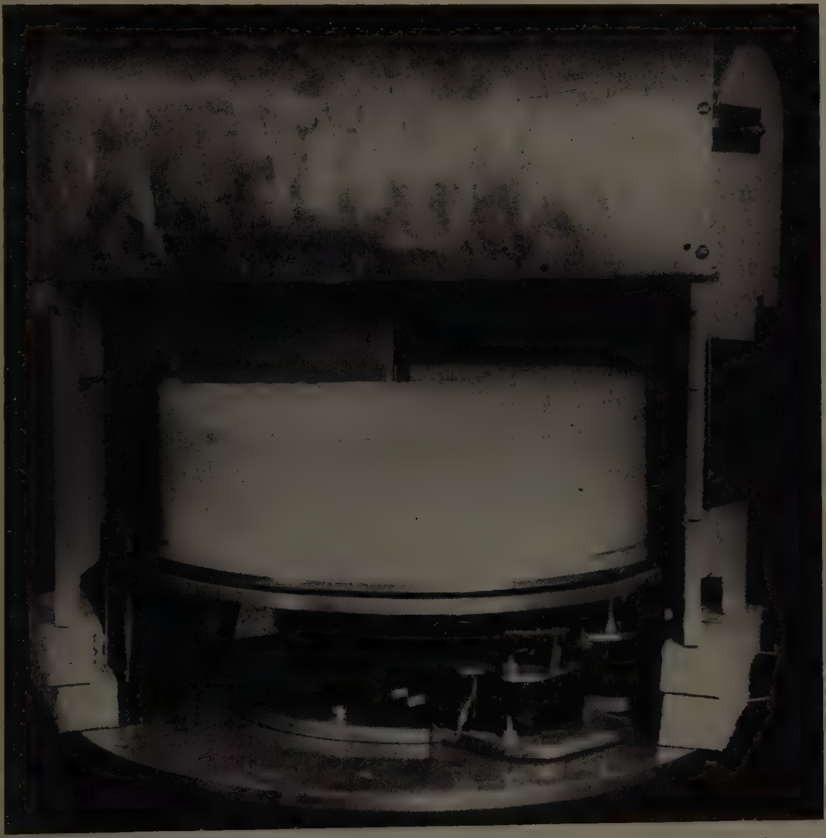

FIGURE 4. Apparatus for the continuous scraping and removal of the dried surface. See text for description.

The scraping knife required some experimentation before a suitable form was developed. Its final form is shown in FIGURE 5. The cutter is held above the surface by a rod until a vacuum has been obtained. This rod is knocked away on the first revolution of the table, so that the cutter falls like a guillotine onto the drying surface. The separated dry material moves down the angle of the knife and is discharged over the edge of the table.

The heat is supplied by 2 tubular electric fire elements set in 2 parabolic aluminium screens arranged as a V, so that no heat is applied directly above the cutter.

Temperature measurements also presented a difficult problem. One thermocouple was placed below the table so that the dry powder fell over it and collected round it, thus giving an index of the highest temperature to which the dried product was raised. The end point of the experiment was indicated best by placing a thermocouple above the drying surface. During drying this surface was cooled by the water vapor coming from the drying surface; at the completion of drying this thermocouple temperature rose rapidly.

It was necessary to make a groove in the bottom plate of the mold; otherwise toward the end of the experiment there was a tendency for the frozen material to separate from it.

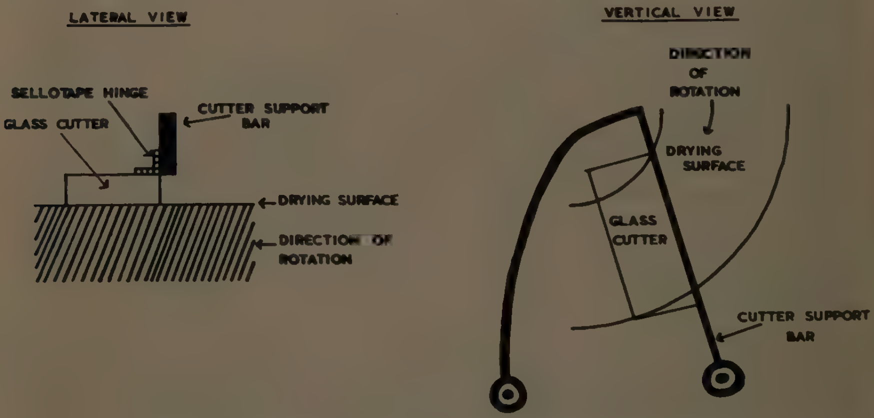

FIGURE 5. Schematic diagram of the surface-cutting mechanism.

Experimental

The experiments were carried out using blood plasma and 20 per cent coffee extract. Starting with a low heat input of 75 w and thicknesses of material of 1 in., heat and depth were gradually increased until a 2½-in. layer was dried in 21 hours with a heat input of 150 w. At this level about one third of the heat input was being lost otherwise than by radiation onto the drying surface, and the refrigerator was fully loaded.

The dried coffee extract was rapidly and completely soluble and had lost no flavor. The dried blood plasma was rapidly and completely soluble; no further tests such as osmotic pressure and electrophoresis have as yet been made on it.

The only obstacles that developed were purely mechanical. Most arose from the prefreezing in the brass mold so that the surface, after freezing, was like a mountain range on which the knife edge jammed, causing stalling of the motor drive. This difficulty was overcome in 3 ways: first, the drive power was increased by using a second motor; second, prefreezing was carried out in

2 stages, the main bulk being first frozen and a thin layer of liquid being added so as to smooth out the peaks; and, third, the design of the knife was modified. In this latter connection, by far the most satisfactory arrangement resulted from hinging a piece of glass ⅛ in. thick and 1 in. wide to the bottom of the cutter blade with a piece of cellulose tape (Sellotape). This glass lies flat while cutting a smooth surface, so that there is no tendency to dig in, but if it meets any obstruction the trailing edge rises and the angle of cut is at once reduced.

At high drying speeds there is a great tendency for the separated dry powder to be carried off in the water-vapor stream. Design should provide for the separation of this powder from the vapor stream if high speeds of drying are required.

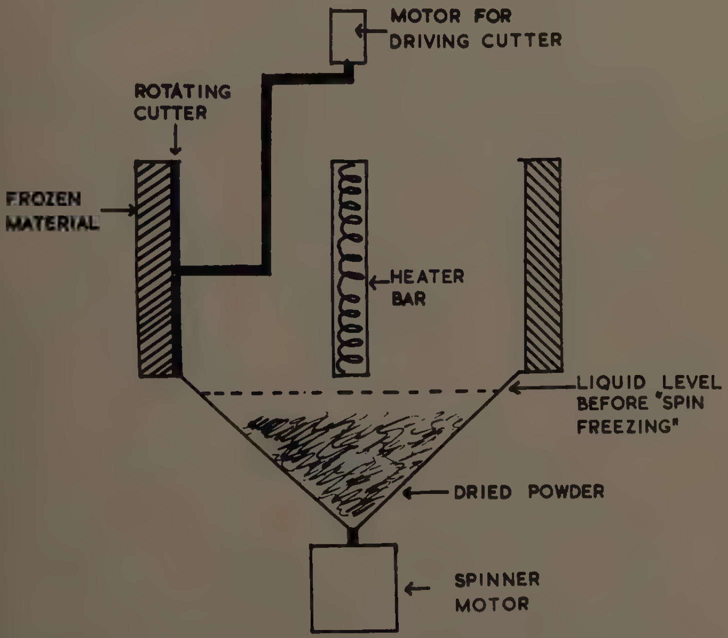

FIGURE 6. Schematic diagram of a vertical spin freeze-dryer arranged for continuous surface-cutting.

In a final design much more attention should be given to radiating the heat only onto the drying surface, for the heating of other structures would limit greatly the very great drying speeds of which this method is capable.

Discussion

The apparatus described in this paper was designed to demonstrate a theory in as simple a way as possible and to use as much existing apparatus as practicable.

FIGURE 6 shows a possible way in which this method could be combined with vacuum spin freezing (Greaves, 1944). This would have one great advantage over straight prefreezing in that a smooth frozen surface would result.

Obviously, this method has no place in the drying of small individual doses in separate ampules, although radiant heat directed onto the surface would always seem preferable to any form of heating from below. Its principal application would seem to be in the drying of large quantities in bulk. High speeds of drying should be obtained in much smaller drying chambers than are currently used.

Perhaps the most fascinating application is in really low-temperature drying. At such temperatures the moment the drying surface recedes so that there is a layer of dried material between it and the condenser, the obstruction becomes such that only minute amounts of heat can be applied, and drying becomes very slow. By keeping the drying surface always at the surface, high speeds of drying become possible even at very low temperatures.

Summary

The theoretical reasons for expecting that a very high rate of drying could be obtained if the drying surface were heated by radiant heat and the dried material continuously removed are outlined.

An apparatus is described for the practical demonstration of the correctness of this theory.

Possible uses for this method of drying are discussed.

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LOW-TEMPERATURE PREPARATION TECHNIQUES FOR ELECTRON MICROSCOPY OF BIOLOGICAL SPECIMENS BASED ON RAPID FREEZING WITH LIQUID HELIUM II*

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With the improved methodology of modern ultrastructure research[1-6] it now appears feasible to study the fundamental mechanisms of biological freezing[7-9,98] at the molecular level. Conversely, the development of adequate preparation procedures is a prerequisite for successful biological application of powerful analytical techniques such as electron microscopy, by means of which the array of atoms in crystalline lattices has already been visualized.[10] Since rapid freezing suspends all physiological activity, immobilizing and preserving labile tissue constituents, low-temperature techniques provide one of the most promising approaches toward reducing the complex preparation artifacts that impose serious limitations on all investigations of the living state. It therefore seems likely that significant advances in the study of life processes under conditions of minimum perturbation will depend to a large extent on further development of the unique potentialities inherent in the low-temperature domain. In turn, electron microscopy, X-ray diffraction, and other physical techniques of molecular biology may well become the key analytical tools for critical evaluation of the basic parameters that determine optimum preservation of frozen biological systems.

Bearing this complementary interrelationship in mind, we shall attempt in the following review to indicate how techniques devised primarily for electron microscopy may be of general interest in the field of low-temperature biology. To illustrate the potentialities of the extremely low temperature range for biological research, we shall survey preliminary results obtained with tissue preparation techniques based on rapid freezing in liquid helium II at 1° to 2° K.[11,12] It is hoped that the described experimental approaches and the underlying theoretical considerations derived from the unique properties of liquid helium II[13] may serve to stimulate further biophysical research in the temperature range close to absolute zero.

Another promising area for investigation is the application of electron microscopy to the direct study of ice crystal nucleation and growth at low temperature and pressure. Thanks to recent technical refinements[14,15] specimen damage due to electron bombardment can be minimized by cooling the suitably insulated specimen support with liquid nitrogen (FIGURE 4) while performing all observations with an electron microbeam of low intensity. Under these favorable conditions submicroscopic ice crystals have been observed directly during all stages of their formation by deposition of water vapor on a refrigerated film

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base (FIGURE 9). Moreover, by stabilizing the thin ice crystals at appropriate low temperatures, both high-resolution electron micrographs and electron diffraction patterns can be recorded from the same selected specimen region (FIGURES 5 and 6). Straightforward identification of the characteristic structural forms of ice at different temperatures is thus possible, confirming the results of previous investigations based primarily on the indirect methods of X-ray diffraction[16,17] and electron diffraction.[18-21] Finally, by oriented deposition of the ice crystals on coherent single-crystal films, typical electron-optical effects such as moiré patterns[22] were recorded on electron micrographs (FIGURE 7). In this way indirect resolution of the atomic arrangement in ice crystals[23] eventually may be achieved, and a detailed investigation of experimentally induced lattice imperfections[24] can also be carried out.

By demonstrating that frozen water can be examined under the extreme conditions of high vacuum and electron bombardment, which had hitherto severely limited the application range of electron microscopy, it now becomes feasible to consider electron optical studies of biological systems in a form more closely approximating their native hydrated state. Therefore, it is not difficult to foresee development of the new field of low-temperature electron microscopy as the high spatial resolution of the electron microscope gradually achieves operational complementarity with an enhanced temporal resolution resulting from sequentially arrested states of activity or from the reduced molecular motion characteristic of freezing.

Applications of Liquid Helium II for Rapid Freezing and Stabilization of Biological Systems at 1° to 2° K

It is commonly assumed that the biochemical and biophysical changes that determine the metabolic rate of organisms are essentially arrested at liquid nitrogen temperatures (−195° C.). Classic low-temperature biology, like most of classic chemistry,[25] has therefore dealt with only a few octaves of temperature; the whole broad range between liquid nitrogen and liquid helium temperatures has been almost entirely neglected.

Recent studies[26-28] clearly indicate that many chemical reactions involving addition of hydrogen atoms, which diffuse readily through solid olefins and other hydrocarbons, can take place at liquid nitrogen temperatures, and even at 20° K,[29] with measurable rates of reaction. Moreover, many unstable chemical species such as free radicals still show considerable reactivity at 70° K, and it has only recently been possible to trap these chemical fragments by freezing them into an inert solid at the extremely low temperatures obtained with liquid helium.[30-32] Free radicals and other transient intermediates with unpaired electron spin play a key role in enzymatic reactions and metabolic electron transfer.[33] The generation of abundant free radicals is inextricably linked with all biological effects of ionizing radiation, and their study is therefore basic to an understanding of radiation damage.[34] Electron-spin resonance as the main tool for the study of free radicals in biological systems has important limitations and lacks the necessary sensitivity for adequate detection of the small, steady-state concentrations of free radicals in many biological processes.[33] These considerations are particularly valid for the field of radiation biology, with which the present investigation is principally concerned. However, it is also becoming increasingly apparent that a closer approximation to the living state requires more than the preservation of the chief organic and inorganic constituents of the cellular organization in tissues, including the predominant water content. In addition, it is obviously essential to preserve a critical population of highly labile, mostly short-lived intermediates and other unstable chemical species that are generally referred to as free radicals.[30]

Eventually, therefore, the stabilization of free radicals within the tissue matrix by rapid or ultrarapid freezing at liquid helium temperatures appears to be essential, in combination with electron-spin resonance and other analytical methods, including electron microscopy, for the study of short-lived intermediates and of free radical interactions in biological systems.

The experimental criteria that have been successfully applied in the production and study of trapped radicals[30-32,35] thus become applicable to biological systems. Essentially, then, the trapping or storage of radicals involves the isolation of these highly reactive intermediates within a matrix of inert material under conditions that will practically eliminate diffusion and recombination; this can be achieved only at liquid helium temperatures.

Considering the widespread occurrence and significance of free radicals, the possibility of achieving their stabilization, and even enrichment,[30,31] in intact biological systems would itself justify the application of extremely low temperatures in biological research. Moreover, although ice retains appreciable amounts of entropy at low temperatures,[36] the far greater stability of the water molecules and the considerable reduction, if not abolition, of chemical reactions at temperatures close to absolute zero might permit almost indefinite preservation of viable organisms. This aspect has in fact been the major incentive hitherto for the biological applications of liquid helium.[37-42]

Aside from being indispensable for attainment of temperatures below 15° K, liquid helium possesses further unique properties and, under certain conditions, it may prove to be more suitable for very rapid cooling of biological specimens than the refrigerants ordinarily employed. Two forms of liquid helium, I and II, are commonly distinguished; of these, the low-temperature phase, called liquid helium II, exhibits the more striking properties, which will be detailed in view of their possible operational significance in low-temperature biology.

Properties of liquid helium II. Normal atmospheric or well helium gas condenses into the "quantum liquid state," which is so unusual that some investigators refer to it as the only representative of a fourth state of matter. The most abundant stable isotope (He_2^4) of this noble gas condenses below its critical point at 5.2° K into a colorless liquid of low density that evaporates quickly because of its extremely small heat of vaporization (93 joules per mole) and must therefore be well protected by surrounding the helium Dewar vessel with liquid hydrogen or liquid nitrogen. At the temperature of 2.19° K, called the λ-point, which marks the transition between phases I and II, liquid helium undergoes a remarkable second-order transformation characterized by sharp discontinuities in the specific heat, thermal conductivity, viscosity, and other physical properties.[13,43,44]

The spectacular nature of this transformation is clearly discernible every time liquid helium II is prepared. When liquid helium I, with its normal thermal conductivity of the order of 10^{-5} watt units (approximately equivalent to that of air at room temperature), is evaporated under reduced pressure it boils violently, with profuse formation of clusters of small bubbles. However, as the temperature falls below the λ-point, all bubbling suddenly ceases and a limpid column of quiescent fluid now fills the inner Dewar vessel (FIGURE 1*a*). In this bath there is no boiling because the million-times-greater thermal conductivity of liquid helium II prevents any temperature differences from arising between bottom and top of the bulk liquid that might be large enough to allow vapor-bubble formation.[44]

Thermal superconductivity is therefore an outstanding property of liquid helium II and, in certain circumstances, the bulk liquid will conduct heat 10,000 times better than will copper, achieving the singularly high conductivity of 10^4 watt units.[13,44] Moreover, this transfer of heat does not conform to the usual law of heat conductivity and depends intimately on the temperature gradient and on the geometry of the measuring container.[13] It is also found that a temperature wave is propagated through the liquid with a finite velocity when a heat pulse of short duration is generated at one point in a bath of helium II.[13,45,46] This traveling temperature wave has been designated "second sound" because heat transmission in liquid helium II is closely analogous to the propagation of ordinary sound waves through an elastic medium.

Although the bulk liquid helium II is a superconductor of heat, it has been found[47,48] that all solid surfaces immersed in the liquid behave as if they were covered by a very thin, poorly conducting boundary layer. Heat transport through this boundary layer increases roughly proportionally to the third power of the temperature for small temperature differences between the solid and the helium bath.[13,48,49] It has been suggested[50] that the thermal boundary resistance (Kapitza jump) between a solid and liquid helium II is due primarily to acoustic mismatch at the interface and also may be related to the microstructure of the solid surface.[51]

Superfluidity is another strange and unique property of helium II that enables the bulk liquid to flow so rapidly through the finest capillaries that it seems to have almost zero viscosity. In fact, the velocity of flow of liquid helium II appears to be approximately inversely proportional to the width of the narrowest channels, which are often less than 1 wavelength of light in diameter and would be completely impervious to helium I. An additional property of the liquid is connected with the formation of a thin film of helium II that migrates rapidly over all contacted surfaces above the bath level. Numerous investigations[13,45,46] have established the fact that practically frictionless transfer of liquid helium II is effected by this mobile film, which is approximately 100 atoms thick and creeps over all solid surfaces that have maintained their temperature below the λ-point.

In order to gain a better understanding of the properties of liquid helium II relevant to the present studies, it is useful to consider salient features of the theoretical ideas concerning the nature of liquid helium. The 2-fluid model of Tisza has been successful in accounting for many of the unique properties of liquid helium II and has provided the most useful basis for experimental research.[13,44-46] According to this model, helium II is considered to be a homogeneous mixture of 2 completely interpenetrating fluids that consist of the same type of particle (helium atoms), but have different heat content and are distin-

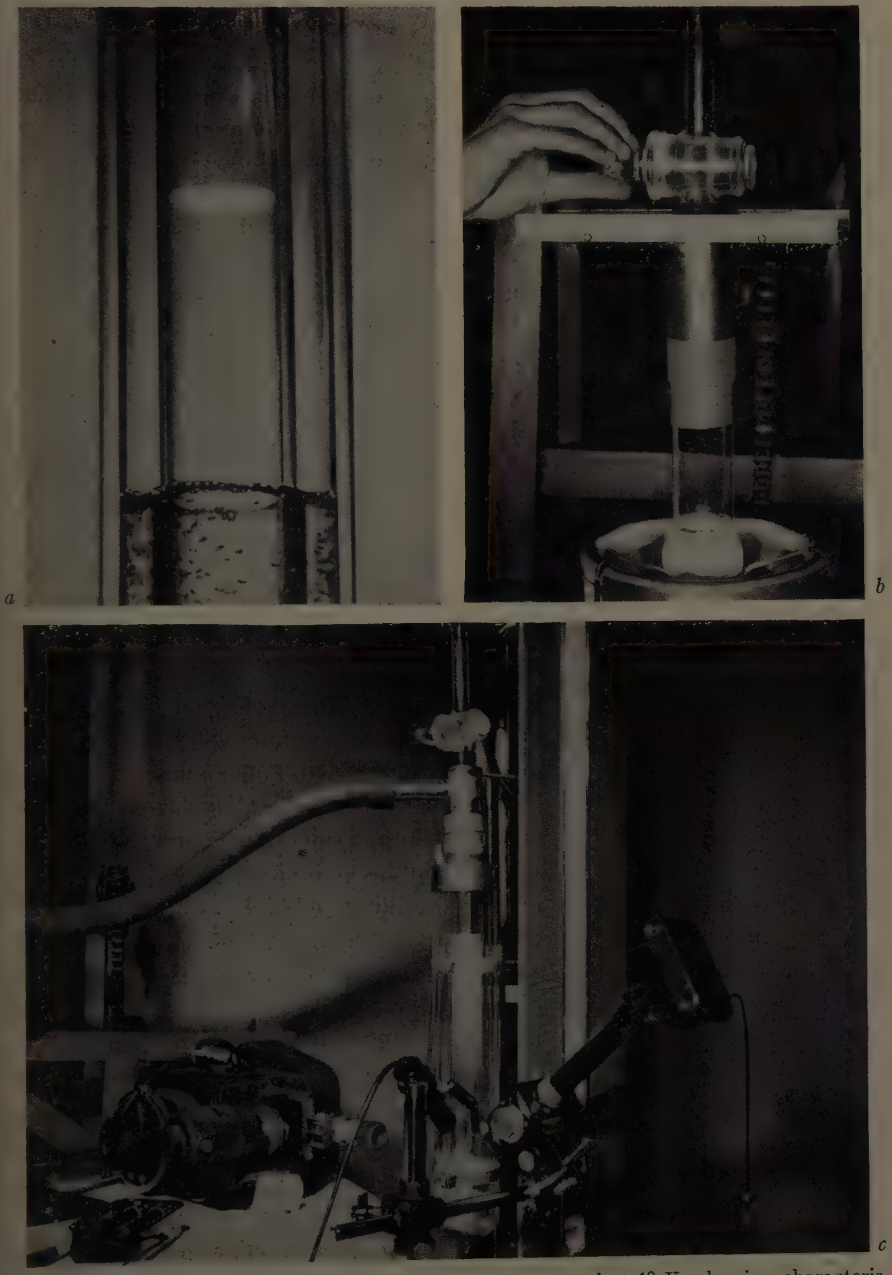

FIGURE 1. (*a*) Liquid helium II in shielded Dewar vessel at 1° K, showing characteristic quiescent appearance. (*b*) Specimen stage for introducing fresh or glycerinated tissues into liquid helium II without breaking the vacuum. (*c*) Equipment for rapid freezing of biological specimens in liquid helium II, with attached gas thermometry and photographic recording devices. Installed at the Cryogenic Engineering Laboratory, Massachusetts Institute of Technology, in cooperation with Samuel Collins.

guished by different hydrodynamic behavior. One of these fluids, called the superfluid component, has zero or vanishingly small viscosity and very small energy; moreover, it is incapable of taking part in dissipation processes. The other normal fluid component retains the properties of an ordinary liquid or viscous gas and possesses nearly all of the thermal energy of the mixture. The superfluid fraction slides freely over all surfaces and through the normal fluid without transferring momentum, the relative proportions of the two fluids depending upon the temperature. The superfluidity phenomena in thin capillaries are explained by assuming that the superfluid component can flow unhindered through the narrow channels, while the normal fluid is prevented by its viscosity from passing through them.

The super heat conductivity can be attributed to a characteristic internal convection mechanism that arises from the different flow velocities of the superfluid and normal components and is connected with a large thermal energy transfer without any mass transfer. This general picture of the complex heat transport process helps to explain the observed dependence of the thermal conductivity of helium II on the temperature gradient. However, as Mendelssohn[13] has pointed out, our knowledge of the entire subject of heat conductivity in helium II is still incomplete; much further work remains to be done.

While the described phenomena are found only under certain conditions and within a restricted temperature range, it is nevertheless worthwhile to consider the potential advantages of liquid helium II over liquid helium I for experimental work in low-temperature biology. In general, the poor thermal conductivity of liquid helium I makes it less suitable as a refrigerant than liquid helium II for direct, rapid cooling of biological specimens to the lowest temperatures. Despite the described boundary resistance[47] impeding heat transfer, rapid and even ultrarapid cooling of suitably thin specimens (that is, sprayed microdroplets) to temperatures close to absolute zero can be obtained with liquid helium II. However, in view of the peculiar nature of heat transport in helium II and the characteristic dependence of the thermal conductivity on the temperature gradient, it may be necessary to effect ultrarapid freezing of certain specimens with liquid nitrogen-Freon mixtures before transfer to the liquid helium II bath. That cooling to temperatures of 1° K and even lower is not in itself detrimental has been established by the pioneering work of Becquerel[37-40] and Lipman,[41] who demonstrated the survival of a wide variety of lower life forms and seeds of higher plants after freezing with liquid helium. Recently Jacob *et al.*[42] have frozen normal human spermatozoa and conjunctival cells treated with 6 per cent glycerol to −272.2° C. in liquid helium and have reported that upon subsequent rapid thawing the conjunctival cells continued to proliferate in tissue cultures; 60 per cent of the spermatozoa were motile, and even without protective glycerol treatment 10 per cent of them maintained motility.

Once the specimen has been cooled below the λ-point, the unique properties of liquid helium II become of greater significance. Thus, the different effects of ionizing radiation on biological specimens immersed in a bath of helium II would be largely determined by local trapping of any free radicals produced within the tissue matrix and also by the far more efficient dissipation of energy mediated through the closely interlinked properties of heat superconductivity and superfluidity. This is of importance, not only in radiogenetic studies, but in all other types of experimental modifications at low temperatures that are compatible with the subsequent survival of organisms.

Based on the foregoing considerations, liquid helium II would appear to represent an ideal refrigerant for rapid or ultrarapid freezing of biological specimens under certain conditions because it is chemically inert and exhibits the unique properties of heat superconductivity and superfluidity at temperatures 100° C. lower than those obtained with the standard liquid nitrogen coolant mixtures.

Immersion of biological specimens in liquid helium II. Pursuing earlier studies with liquid helium I,[52] a series of preliminary biological experiments with liquid helium II have been conducted during the past year in collaboration with Samuel C. Collins and his associates at the Cryogenic Engineering Laboratory of the Massachusetts Institute of Technology. As shown in FIGURE 1*b* and *c*, the equipment for immersion of specimens in the helium II bulk liquid consists essentially of a shielded helium Dewar vessel with an attached simple gas thermometer fitted with a special specimen stage; the vessel can be evacuated by a large forepump of 311 cu. ft./min. capacity. A high-speed camera for photographic recording of the immersion process and a stereoscopic microscope for observation of the specimens within the helium bath complete the basic arrangement. The principal practical problem of introducing the specimen into the liquid helium II bath under reduced pressure without breaking the vacuum was solved by a simple type of stage (FIGURE 1*b*) consisting of a large glass stopcock provided with a tubular extension for insertion of the specimens. After filling the inner Dewar vessel with liquid helium I, a sufficient quantity of the helium II bulk liquid is produced by rapidly lowering the vapor pressure to approximately 70 μ, which is equivalent to $-272.2°$ C. In view of its small heat of vaporization, relatively large amounts of liquid helium II must be provided for processing even a limited number of specimens. The suitably protected specimen lodged in the tube, which can be partially evacuated or filled with helium gas, rapidly falls into the liquid helium II bath when the stopper is turned (FIGURE 1*b*).

The stage can be readily adapted for precooling of the specimen with liquid nitrogen or liquid hydrogen, and it is also possible to inject microdroplets of cell suspensions directly into the helium II bath by means of an attached spraying device. Full details of the experimental procedures are described elsewhere. In a typical experiment fresh or glycerinated thin tissue specimens (approximately 50 to 100 μ thick and a few millimeters long) are immersed in the liquid helium II without appreciable rise in temperature or modification of the bath. Preliminary studies of the immersion process carried out by means of high-speed photography (1000 to 4000 frames per second—*see* FIGURE 2) in collaboration with D. Eldridge of the Electrical Engineering Department of the Massachusetts Institute of Technology show that, despite considerable turbulence at the surface of the bath, no bubble formation occurs. This phenomenon indicates that equilibration of the temperature difference is exceedingly rapid. However, it is necessary to carry out more exact determination of the cooling velocities achieved with the different types of specimens, employing essentially the techniques described by Luyet and Gonzales.[53,97]

Thin specimens within the helium II bath show only minor warping and are frequently transparent, while thicker specimens reveal opaque areas alternating with clear regions. The better preservation of fine structure as compared with control specimens rapidly frozen in Freon-liquid nitrogen mixtures is evident only upon electron microscopic examination of thin specimen sections prepared according to the low-temperature techniques described below.

Certain microorganisms and other specimens transparent to the electron beam can be examined directly by shadow-casting of the microdroplets[54] frozen under the favorable conditions encountered at liquid helium temperatures. However, in order to obtain accurate information on the ultrastructure of viable tissues at temperatures approaching absolute zero, it would be necessary to perform X-ray diffraction studies and related investigations, using specially designed low-temperature stages. The variety of experiments that can be performed with the comparatively simple equipment described here will give some indication of the promising approaches offered in this temperature range.

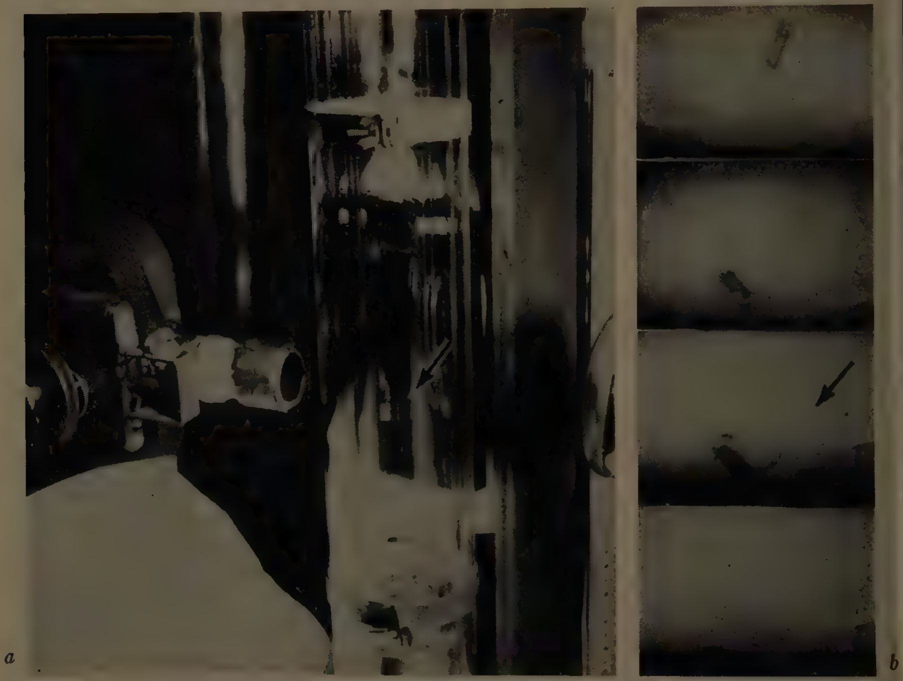

FIGURE 2. (*a*) High-speed camera for recording the immersion of specimens in helium II bulk liquid (*arrow*). (*b*) Serial high-speed photographs, showing immersion of specimen in liquid helium II without producing boiling (*arrow*). Photographs reproduced by courtesy of H. E. Edgerton of the Massachusetts Institute of Technology.

Potential Biophysical Applications of Liquid Helium II

Beyond its usefulness in the stabilization of free radicals in biological systems there are numerous problems of practical significance connected with liquid helium II. For example, the problem of establishing the ability of organisms to survive almost indefinitely at sufficiently low temperatures is of major importance. Although no detectable metabolic activity has been reported at liquid nitrogen temperatures, conditions verging upon unlimited conservation of viability can be expected only close to absolute zero at liquid helium temperatures, for which an incomparably greater reduction in metabolic rate (approximately 10^{13} times) have been calculated.[40] Any attempt to determine empirically the duration of survival of organisms at such low temperatures would involve prohibitively long time factors, and it is principally through the use of refined analytical techniques such as X-ray diffraction and electron microscopy that we may hope to establish whether "indefinite" preservation of biological systems is actually possible as we approach the realm of vanishing entropy at absolute zero.

The study of organisms at these temperatures is also of importance in connection with the new field of space biology, as already realized by Becquerel.[37-40] Experimental duplication of many conditions found in outer space, as well as of primeval states that might have played a role in the origin of life on our planet, are accessible through investigations in this temperature domain.

Of further significance in connection with attainment of extremely low temperatures using liquid helium II is the demonstration by Powers and his coworkers[55,56] of a transition region in the temperature dependency of radiation inactivation of dry bacterial spores at about 130° K, characterized by temperature independence of radiation sensitivity below 125° to 130° K. If the indicated complete temperature independence of radiation sensitivity below this range can be established for other types of cells, the conservation of biological systems at low temperatures would become of key operational importance. It has already been shown by Parkes[57] that low temperature (−75° C.) "affords a substantial protection against the effects of x-irradiation" (page 524). Provided that conditions for adequate survival of biological systems after freezing to liquid helium II temperatures can be established, the degree of protection against high dosages of ionizing radiation at temperatures of 1° to 2° K may be expected to be much greater than that obtained at liquid nitrogen temperatures. It thus appears feasible to set up storage banks of important cells, including bone marrow, whole blood, and even spermatozoa and ova which, maintained at liquid helium temperatures and adequately shielded, would be almost entirely impervious to ionizing radiation in case of atomic catastrophe, and might then be available for effecting critical transplantations and even "genetic transfusions" in future generations.

Liquid helium II should also prove useful as a powerful experimental tool in many other biochemical and biophysical studies. Now that liquid helium is being used in greater quantities in the field of masers, superconducting circuits, and other areas of applied research, the practical difficulties connected with its use are largely obviated. The techniques of handling liquid helium are easily mastered.[58,59] Moreover, the far greater safety of liquid helium as compared with the isopentane-propane-liquid nitrogen mixtures, or with liquid hydrogen, would constitute a decisive factor in determining the choice of this refrigerant for many biological experiments.

Systematic research in the field of cryobiology may reveal new phenomena derived from the cumulative effects frequently associated with the high degree of molecular order which gives rise to the remarkable solid state induced in biological specimens at temperatures close to absolute zero.

Improved Low-Temperature Preparation Techniques for Electron Microscopy

Although the standard freeze-drying[60,61] and freeze-substitution techniques[62-66] have been used successfully in the field of light microscopy, their application to electron microscopy of biological tissues[67,68] has been disappointing, since the degree of morphologic preservation attained is generally inferior to that of the standard preparation techniques. This is due to numerous factors that have not been adequately investigated. The fact that ice crystals can still grow within the specimen[69,70] and thereby produce serious tissue damage during the comparatively long periods of dehydration at temperatures above the critical crystallization point of water (−120° to −130° C.) has not been adequately taken into account in the conventional freeze-drying and freeze-substitution methods. Likewise, the considerable extraction introduced by impregnation with monomers and during polymerization at temperatures of 40° to 60° C. after conventional freeze substitution, has been another major source of artifacts.

Based on previous investigations that I have carried out during the past 10 years,[71-73] and taking into account recent contributions,[66,74,75] improved low-temperature preparation techniques for electron microscopy[12] have now been developed that consistently yield better morphologic and histochemical preservation than any of the standard freeze-drying or freeze-substitution methods. The new "cryofixation" techniques are based essentially upon the combined application of: (1) rapid freezing of fresh or glycerinated tissues, with liquid helium II at 1° to 2° K; (2) subsequent staining at −150° C. with halogens or organometallic compounds dissolved in isopentane; (3) substitution of the ice matrix in alcohols, acetone mixtures with alkyl halides, or certain organometallic compounds at temperatures of −130° to −80° C.; and (4) infiltration with suitable acrylic monomers and final embedding of the specimens by photopolymerization with ultraviolet light at low temperatures (−80° to −30° C.). This exceptional combination of favorable conditions, minimizing ice crystal formation, artificial osmotic gradients, and extraction artifacts during dehydration and embedding at low temperatures, accounts largely for the superior preservation of macromolecular structure achieved with the improved preparation procedures.[76]

Pretreatment with glycerol has been shown, in agreement with the available information[77-80,100] on its protective action, to preserve the fine structure of tissues at low temperatures as determined by X-ray diffraction and electron microscopy.[76] A technical variant involving controlled glycerination of tissues (30 to 60 per cent glycerol-Veronal buffer solutions) prior to immersion in the refrigerant, followed by freeze-substitution with mixtures of alcohols and acetone at temperatures around −130° C., permits preservation of even relatively large specimens with a minimum of chemical fixation in a condition approximating the native state.

With the new techniques, for example, excellent morphologic preservation of the retina has been obtained. The transversely arranged layered structure of the external retinal rod segments appears to be very compact and, in light-adapted retinas, the constituent unit disks display an intermediate dense line 15 to 20 Å thick (FIGURE 3) with associated granular material that had not been detected previously between the double layers.[11] The cell nucleus, the nerve myelin sheath, and other cytoplasmic components are likewise well preserved. Specimens frozen in liquid helium II generally show a degree of preservation superior to that of those frozen in liquid nitrogen or liquid nitrogen-Freon mixtures. This is presumably due to the smaller size of ice crystals produced by rapid freezing in liquid helium II and to other stabilizing effects at low temperatures that remain to be investigated.

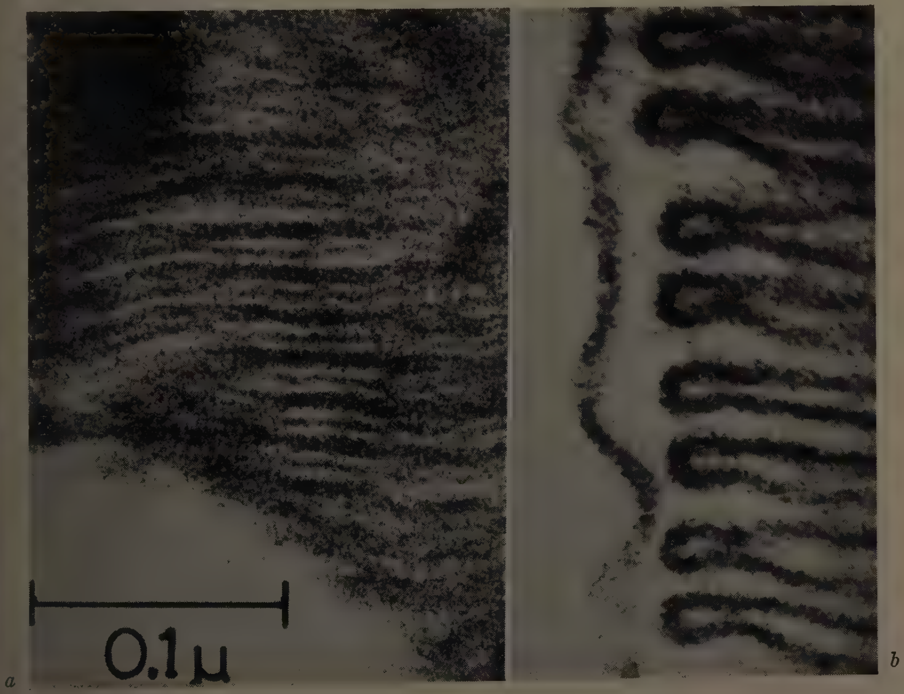

FIGURE 3. (*a*) Electron micrograph of outer segment of retinal rod from light-adapted guinea pig retina frozen in liquid helium II, showing new structural details of lamellar components that are not preserved in corresponding standard preparation (*b*). ×320,000.

Thin sections of the undenatured specimens can be subjected to controlled extraction, enzymatic digestion, and staining procedures and are also suitable for application of high-resolution autoradiography. The use of radioactive tracers in connection with low-temperature preparation techniques appears to be particularly rewarding, since through rapid freezing a high degree of molecular stabilization is achieved, and the tracer itself is immobilized in a position equivalent to that which it had in the living organism. One of the most promising approaches is represented by the introduction of compounds leading to the formation of silver bromide during the process of freeze substitution. The cellular matrix thus substitutes for the gelatin base of ordinary photographic emulsions. By working at low temperatures all interfering chemical activity is considerably decreased during the prolonged exposure periods of several weeks, thereby reducing the fog and background effects without impairing the definition of the autoradiogram. After suitable exposures, the latent image centers marking the sites of β emission of the tritium-labeled compounds are developed and enlarged by using gold-thiocyanate solutions.[76]

These preparation techniques are still in an early stage of development and can be considerably improved, particularly by application of addition polymerization reactions at lower temperatures.

Direct Study of Ice Crystals by Low-Temperature Electron Microscopy

The study of ice crystals by electron microscopy has heretofore been limited to investigations based on replication techniques,[81-86,99] since direct examination of frozen aqueous substances was precluded by vacuum sublimation of the ice and the heating effects of the electron beam under normal operating conditions. However, with recent technical refinements, it is now possible to stabilize ice crystals deposited on a cold substrate by maintaining them at low temperatures during observation in the electron microscope with a microbeam of very low intensity. Under these new experimental conditions supplementary data have been obtained on ice crystal structure and growth.

Previous X-ray diffraction studies[16,17] and calorimetric[20] and electron diffraction investigations[18,19,21,87] have revealed the existence of several structural forms of ice, depending on the temperature. Three types of diffraction patterns were found by Blackman and Lisgarten[21] in their comprehensive electron diffraction studies of deposits formed by injecting water vapor onto a base at low temperature. Below −140° C. diffuse rings were recorded; between −140° and −120° C., a sharp ring pattern characteristic of the diamond-type cubic pattern was seen; and above −100° C. sharp rings corresponding to the normal hexagonal pattern of ice were obtained. It appeared worthwhile, therefore, to try to verify the existence of these 3 structural forms of ice by direct observation of the deposits and correlation with the corresponding electron diffraction patterns.

As shown in FIGURE 4*a*, an object-cooling device after the design of Leisegang[15,88] was used to maintain the thermally insulated specimen holder (FIGURE 4*b*) within the Siemens Elmiskop I microscope at reproducible temperatures ranging between −70° and −120° C. by contact with a cooling finger of copper immersed in liquid nitrogen. The residual or injected water vapor condenses on the cold film mounted on a specimen grid that is protected by a modified aperture device (FIGURE 4*b*, *arrow*) from excessive contamination with diffusion pump vapors. Multiple objective apertures[89] that can be readily exchanged without breaking the vacuum were also required in view of the high contamination rates. All observations were made with an electron microbeam of extremely low intensity, usually covering a specimen area 5 to 10 μ in diameter. This proved to be a critical factor even at low temperatures, since it was invariably found, in agreement with Keller and O'Connor,[14] that irradiation with an electron beam of normal intensity will destroy the crystallinity and diffracting power of sensitive specimens.

Examination of ice crystals deposited on Formvar films. In a first series of

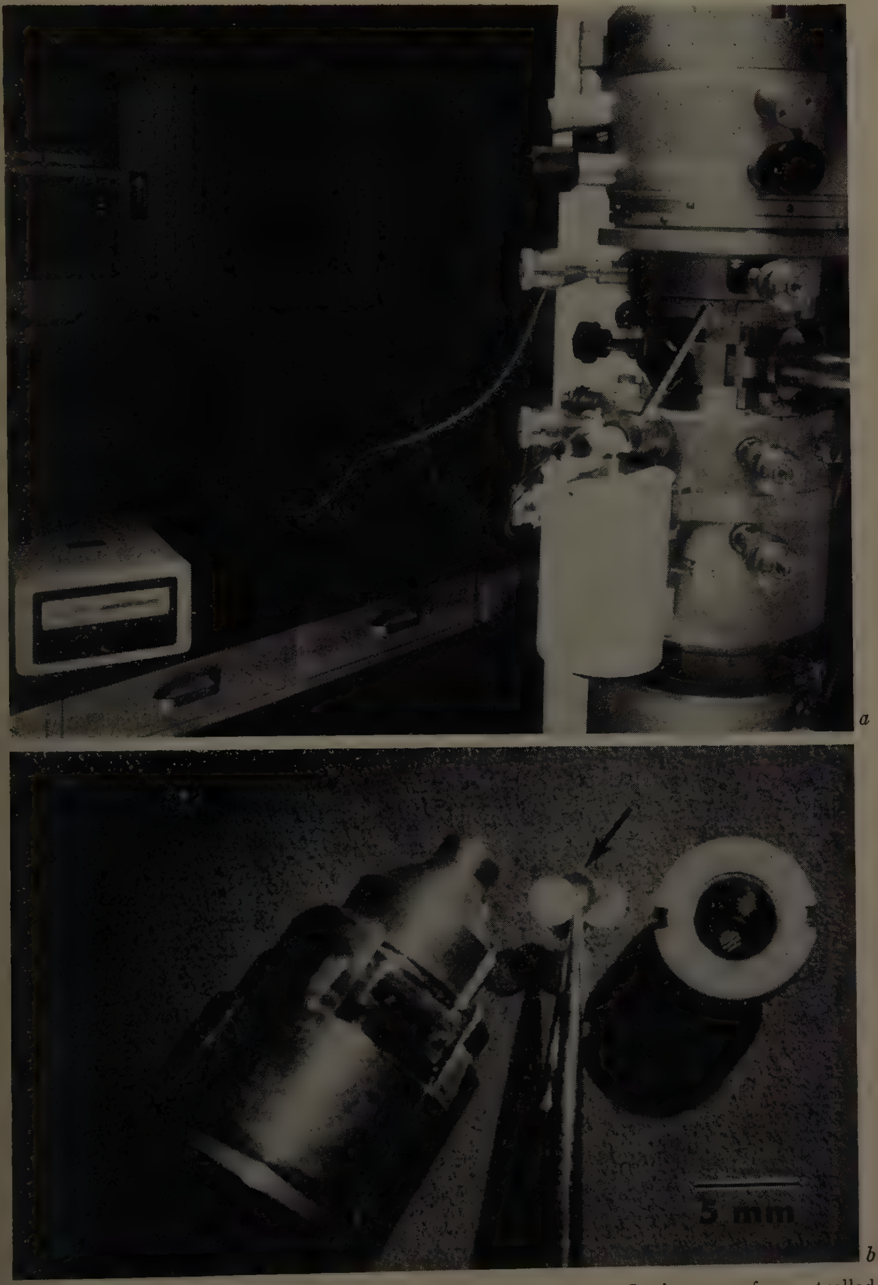

Figure 4. (*a*) Liquid nitrogen cold stage of Siemens Elmiskop I microscope for controlled cooling of specimen holder from −10° to −120° C. during electron microscope observation. (*b*) Specimen holder of Elmiskop I cold stage, showing thermocouple contact and specially designed shielding device, which consists of superimposed copper screens to prevent the condensation of vapors on cooled specimen film.

experiments the Formvar films examined at temperatures ranging from 20° to −70° C. exhibited only the normal background structure, with the corresponding transmission electron diffraction patterns of the usual diffuse-ring type. When the substrate is cooled rapidly to −120° C. the residual water vapor in the microscope column will condense in the form of minute dense particles that are already perceptible before clear-cut changes in the diffuse ring pattern can be detected. However, as these particles become more numerous and grow rapidly in size they can be identified readily as ice crystals by recording the corresponding selected-area diffraction patterns, which now show a striking transformation into the sharp-ring type. The crystals are predominantly cubical in shape (FIGURE 5*a*, *b*, and *c*) at −120° C. and give diffraction patterns that are generally of mixed form, but show a certain predominance of the diamond-type cubic pattern (FIGURE 5*d*) first described by König.[18,19] With only a slight rise in temperature the hexagonal forms become increasingly noticeable and, at −100° to −90° C., the hexagonal crystals are most frequently encountered in the electron micrographs (FIGURE 6*a*), yielding distinct, sharp-ring diffractions corresponding to the normal hexagonal pattern of ice (FIGURE 6*b*). The common occurrence of mixtures of cubic and hexagonal ice in these preparations is in general agreement with the results of previous electron[21,87] and X-ray diffraction studies, taking into consideration that even at temperatures appreciably lower (−190° C.) than those attainable with the present cold stage (−120° C.), cubic ice was never obtained free from hexagonal ice. Perhaps of greater significance is the direct visualization of the postulated ice-crystal forms at different temperatures and the accurate correlation that can now be made with the corresponding electron diffraction patterns, thus dispelling some of the uncertainties introduced by the contamination problem.

Moiré patterns from ice crystals. The submicroscopic ice flakes consistently exhibit extinction contours (FIGURES 6*a*, 8*b*, and 9*b*), reflection images, and other electron optical phenomena typical of thin crystals. Occasionally, when 2 ice crystals overlap, the electron microscope images display regular fringe patterns that may be interpreted as a moiré pattern arising from the coincidence of the projected planes of atoms in the overlapping lattices.[22,24] By means of such moiré patterns indirect resolution of the atomic array in crystalline lattices has been achieved, and the lattice imperfections can also be studied.[10,22,24] Moreover, Dowell *et al.*[23] have indicated that if "a crystal can be superimposed on an isomorphous crystal containing a heavy atom, the moiré pattern should then closely resemble the actual crystal distribution" (page 197).

In view of these interesting possibilities an attempt has been made to obtain moiré patterns in a more controlled fashion by letting the ice crystals grow directly on a suitable single-crystal substrate at low temperatures. Thus, for the cubic type of ice modification, coherent single-crystal films of gold prepared according to the technique described by Bassett *et al.*[22] were selected as an appropriate substrate. The more common hexagonal ice-crystal type has been grown on thin single-crystal lamellae of mica prepared by an improved cleaving method, and attempts to use several types of hexagonal single crystals containing a heavy atom have also been made. These experiments are described

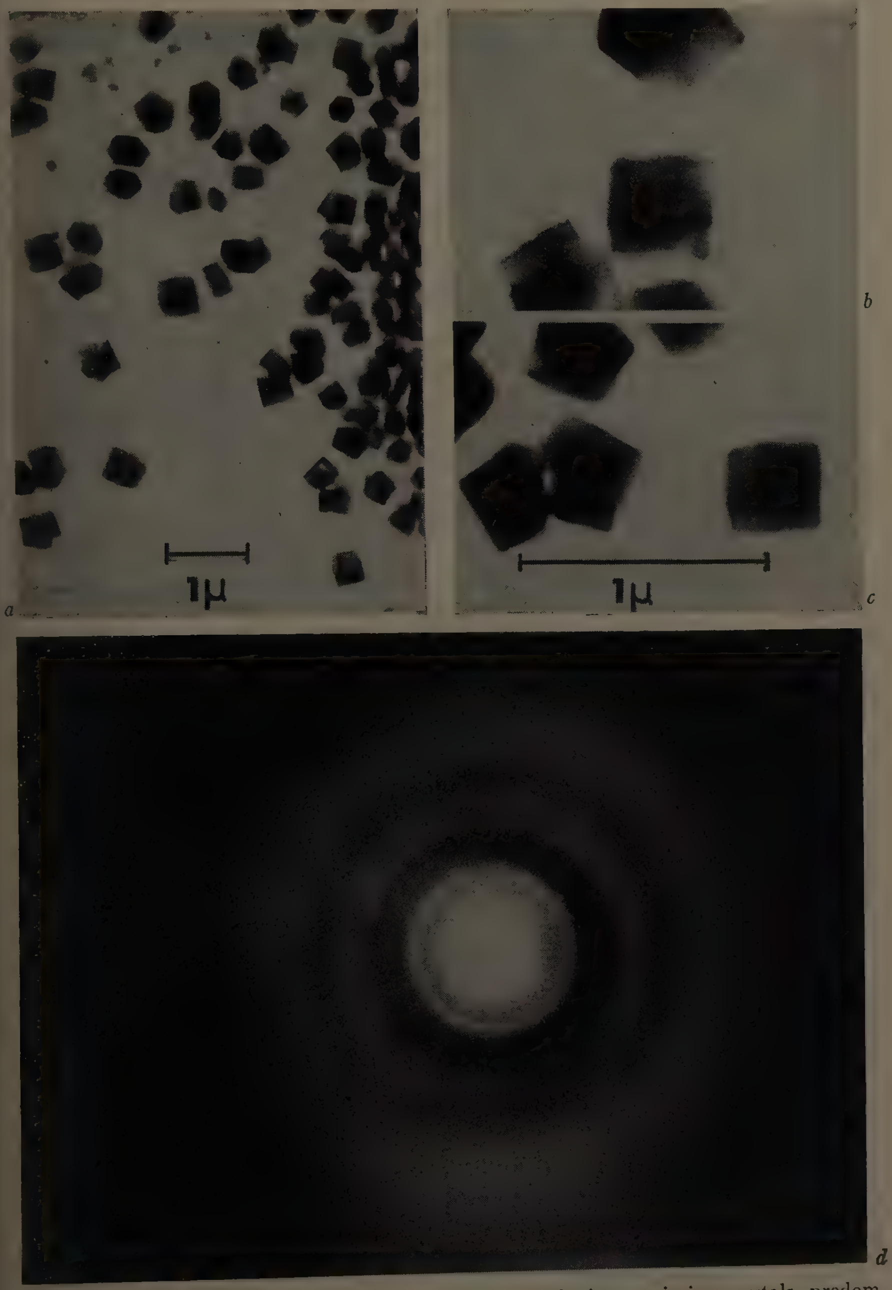

FIGURE 5. (*a*, *b*, and *c*) Electron micrographs of submicroscopic ice crystals, predominantly of the cubic type, deposited on thin film at −120° C. (*a*) ×10,000; (*b* and *c*) ×32,000. (*d*) Electron diffraction pattern of the predominant cubic ring pattern type, recorded with a low-intensity microbeam at −120° C. from the area shown in *a*.

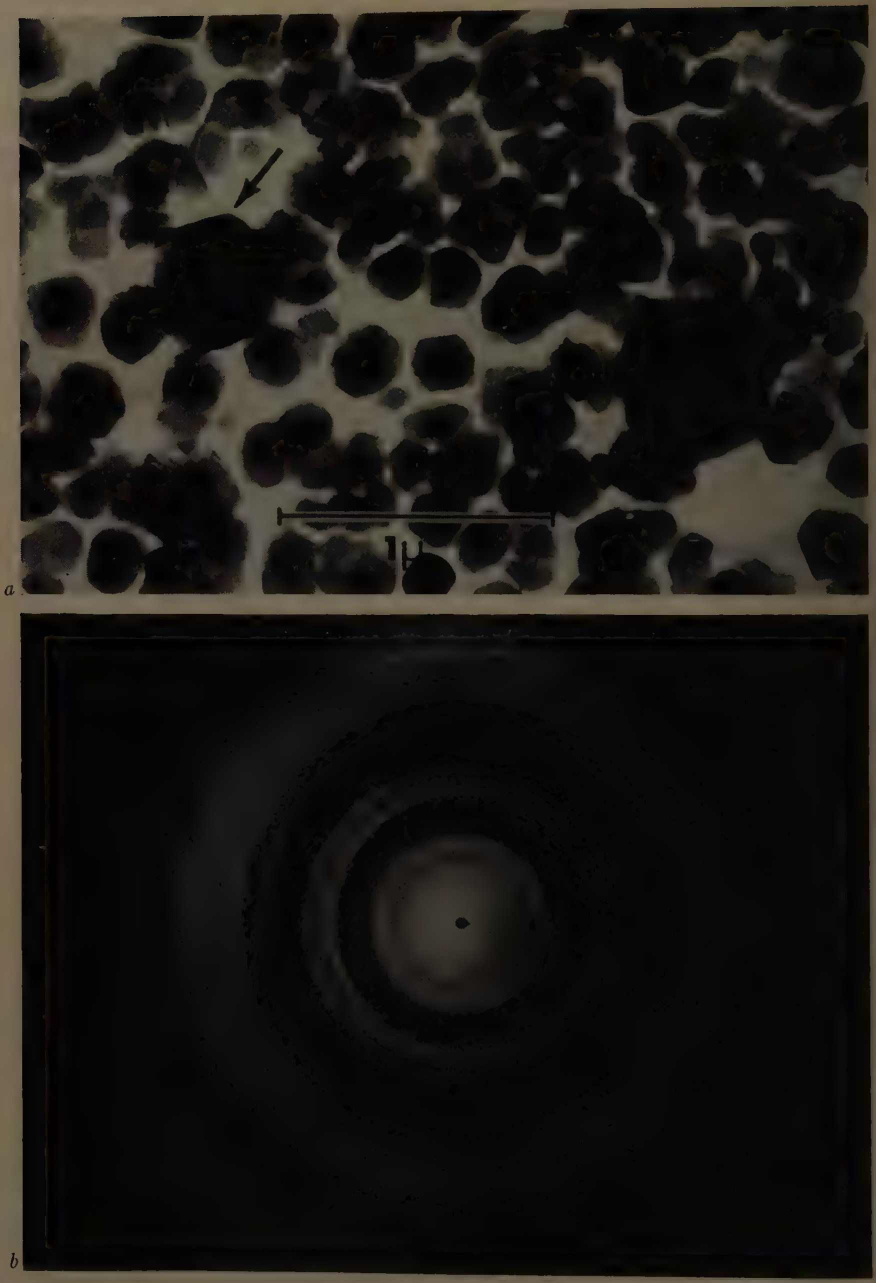

FIGURE 6. (*a*) Electron micrograph of predominantly hexagonal ice crystals (*arrow*) deposited on a Formvar film and recorded at −90° C. ×35,000. (*b*) Electron diffraction pattern of the predominantly hexagonal ring pattern-type, recorded with a low-intensity microbeam at −90° C. from the area shown in *a*.

in detail elsewhere, and only a representative example of the results will be presented in this review.

When ice crystals are grown at −90° to −80° C. on the mica single crystals (FIGURE 7*a*) it is possible to detect, in suitably oriented ice crystals, a series of dense fringes with regular spacings from 15 Å upward. At higher temperatures, when there is coalescence of adjacent ice crystals, these moiré patterns cover wide areas of the specimen (FIGURE 7*b*). The corresponding electron diffraction patterns show the typical arrangement of double-diffraction spots around the primary diffraction spots.[22] Although interpretation of these moiré patterns is still in a preliminary stage, it is hoped eventually to observe patterns that are related more directly to the lattice structure of the ice crystals. It may also be possible to recognize lattice dislocations induced in the ice crystals by irradiation at low temperatures.

Ice-crystal replicas. The practical significance of the contamination problem in these studies becomes evident upon following the melting of ice crystals (FIGURE 8*a*) at temperatures below −70° C. after prolonged irradiation with an electron beam of low intensity. It is then seen that tenuous surface replicas (FIGURE 8*b*) or "ghosts" are left behind at the sites formerly occupied by the individual ice crystals. These ice-crystal replicas result from the deposition of a thin layer of carbonaceous material that is presumably formed by the interaction of the electron beam with the oil-diffusion vapors at the surface of the ice lattice. The replicas are very delicate and seem to conform quite accurately to the finest details of the ice surface structure. Most of the replicas exhibit a regular striated structure (FIGURE 8*c* and *d*) with an average spacing of 10 to 20 Å. Although the possibility of wrinkling during the sublimation process and other artifact sources must be carefully excluded, it will be interesting to compare this type of surface structure with the results of the moiré pattern investigations.

Investigation of ice-crystal growth. The described experimental approaches appear to be well suited for a critical investigation of the early stages of ice-crystal growth[98] and the possible relationship with the postulated "amorphous" or "vitreous" ice modification at the lowest temperatures. When a thin Formvar film is rapidly cooled to −120° C. the earliest stages of deposition are characterized by the appearance of exceedingly small particles, approximately 20 to 50 Å in diameter, which rapidly form larger aggregates of 50 to 200 Å (FIGURE 9*a*), covering the entire surface as the temperature gradually rises. The selected-area electron diffraction patterns are initially of the diffuse ring type, but sharp rings begin to appear when the particles become larger (50 to 100 Å) and exhibit unmistakable signs of crystallinity. Interpretation of these results is beset by the same difficulties that have made it impossible to establish whether the deposits giving rise to the "diffuse ring patterns"[16-21,87] consist of very small ice crystals or of vitreous ice. Within a relatively short time of at most a few minutes, these minute ice crystals grow rapidly (FIGURE 9*b*), approaching micron size, in agreement with the previous investigations of Meryman[81] and others.[86]

In another type of experiment designed to study directly the effects of nucleation on ice-crystal growth, a polycrystalline film of silver iodide was cooled rapidly to −100° C. The ice crystals (FIGURE 9*c*) formed initially were larger

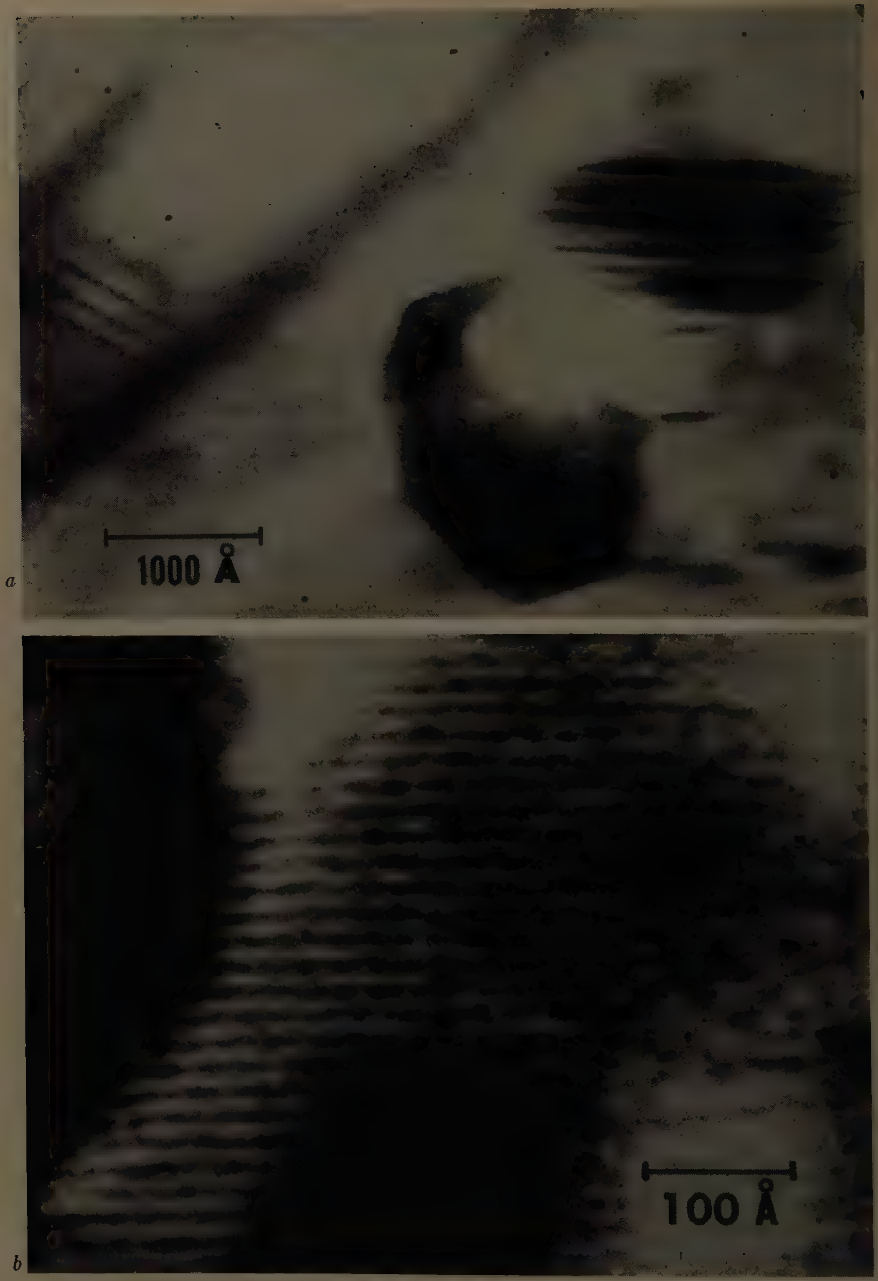

FIGURE 7. High-resolution electron micrograph, recorded at −90° C., of hexagonal ice crystals grown on a thin single-crystal film of mica, showing characteristic moiré patterns. ×200,000. (*b*) Moiré pattern exhibited by ice crystals grown on a single-crystal mica film at −80° C., with a regular period of 18 Å. ×1,800,000.

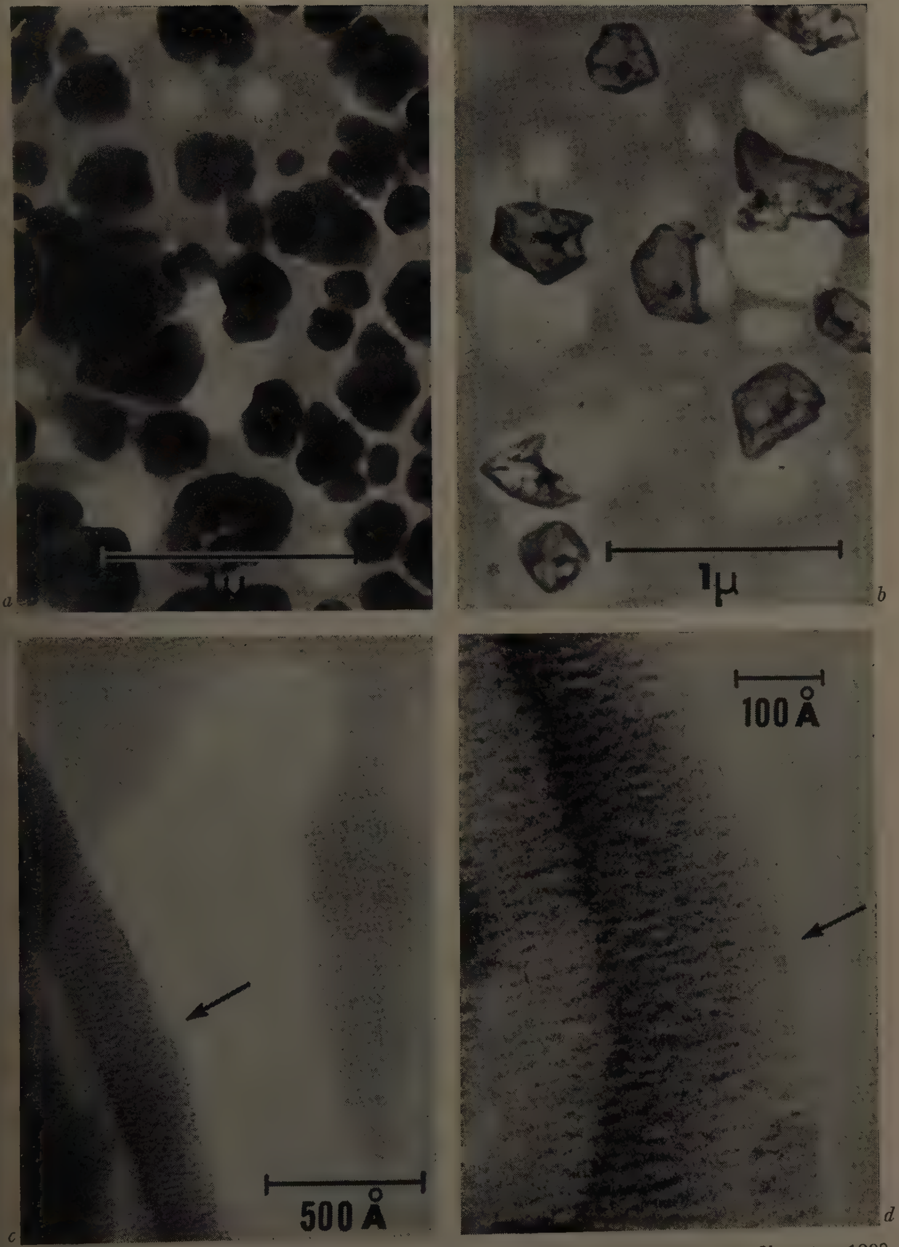

FIGURE 8. (*a*) Electron micrograph of ice crystals deposited on Formvar film at −100° C. after prolonged irradiation with a low-intensity electron beam. ×32,000. (*b*) Electron micrograph of the surface replicas or "ghosts" formed by the deposition of carbonaceous materials on the surface of the ice crystals during irradiation in the electron microscope. The numerous folds in the replicas are caused by collapse after the evaporation of the ice crystal. ×30,000. (*c*) High-resolution electron micrograph of a surface replica (same specimen as in *b*), showing the characteristic striated surface structure (*arrow*), which is particularly noticeable in areas that lie free over holes in the supporting film. ×400,000. (*d*) High-resolution electron micrograph of the ice-crystal replica shown in *c*, showing the regular striated surface structure (*arrow*), which has a periodicity of approximately 10 to 20 Å. ×1,100,000.

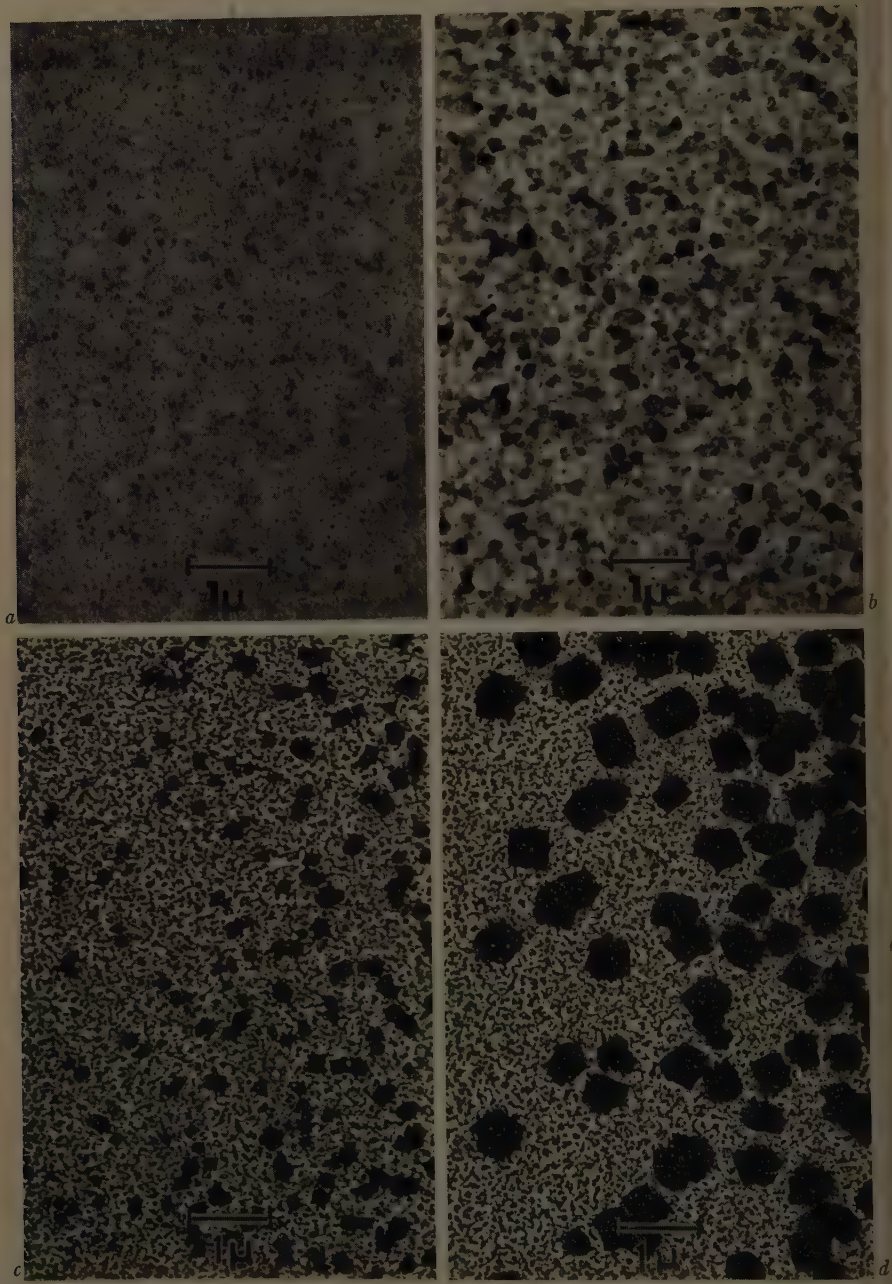

FIGURE 9. (*a*) Electron micrograph of initial ice-crystal formation on thin Formvar film at −100° C. ×10,000. (*b*) Electron micrograph of ice-crystal deposit formed on specimen shown in *a* taken 4 min. later. ×10,000. (*c*) Electron micrograph of initial ice-crystal formation on thin silver iodide film at −100° C. ×10,000. (*d*) Electron micrograph of a corresponding region of the specimen shown in *c* taken 4 min. later to demonstrate the higher rate of ice-crystal growth under favorable nucleation conditions. ×10,000.

than the corresponding crystals grown on an amorphous Formvar film (FIGURE 9*a*); as equivalent time intervals elapsed, the difference in crystal size became even more marked (FIGURE 9*b* and *d*). This direct demonstration of enhanced ice-crystal growth in the presence of submicroscopic nucleation centers may contribute to a better understanding of the artifacts associated with the abnormal electrolyte concentrations induced during the process of freezing. In addition to their deleterious osmotic effects, these artificial electrolyte gradients might act as nucleation centers giving rise to increased ice-crystal damage.

In conclusion, it may be stated that elucidation of the fundamental role played by water in living organisms, taking into consideration the available information on water structure,[90-92] hydration of the proton in aqueous solution,[93,94] and other physicochemical properties relevant to hydrated biological systems, represents one of the most challenging tasks faced by workers in electron microscopy, X-ray diffraction, and other biophysical methods of ultrastructure research at low temperatures.[76,95,96]

Summary

Low-temperature preparation techniques have been developed that give better morphologic and cytochemical preservation of biological specimens than do the standard freeze-drying and freeze-substitution methods. Although intended primarily for electron microscopy, the approaches embodied in these techniques are potentially suitable for long-term preservation of biological systems. The new cryofixation techniques are based essentially on combined application of: (1) rapid freezing of fresh or glycerinated tissues with liquid helium II at 1° to 2° K; (2) subsequent staining at −150° C., with halogens or organometallic compounds dissolved in isopentane; (3) substitution of the ice matrix in alcohols, acetone mixtures with alkyl halides, or certain organometallic compounds at temperatures of −130° to −80° C., and (4) infiltration with suitable acrylic monomers and final embedding of the specimens by photopolymerization with ultraviolet light at low temperatures (−80° to −30° C.), thus permeating the macromolecular matrix of the specimen and permanently stabilizing its fine structure over a wide range of temperatures. Preliminary applications of high-resolution autoradiography techniques for electron microscopy at low temperatures are discussed.

Liquid helium II, the low-temperature form of liquid helium, represents an ideal medium for ultrarapid freezing of thin specimens under certain conditions because it is a superconductor of heat and a superfluid at temperatures 100° C. lower than those obtained with isopentane-liquid nitrogen coolants. Although transfer of heat from the specimen into the bulk liquid helium II is impeded by a poorly conducting boundary layer, exceptionally high cooling velocities are obtained with suitably thin specimens and sprayed microdroplets. In addition, liquid helium II is chemically inert, and thus particularly suited for the stabilization of free radicals and other highly reactive components in biological systems. Beyond its applications in the investigation of serially arrested states of activity in biological systems, ultrarapid cooling with liquid helium II should prove useful in many other biochemical and biophysical studies, (such as free radical and radiogenetics research), that require the attainment of very low temperatures. Systematic research in the field of

cryobiology may reveal new phenomena derived from the cumulative effects frequently associated with the high degree of molecular order characteristic of the solid state induced in biological specimens at temperatures close to absolute zero.

Preliminary results have been described of a direct study of ice-crystal structure and growth by low-temperature electron microscopy, using a liquid nitrogen stage for cooling of the specimen during observation in the microscope with a microbeam of very low intensity. The existence of the cubic and hexagonal forms of ice could be verified by electron microscope observations of the ice crystals and correlation with the corresponding electron diffraction patterns obtained at appropriate low temperatures. By oriented deposition of ice crystals on coherent single-crystal films of mica and of certain heavy metals, characteristic moiré patterns were recorded on electron micrographs. In this way indirect resolution of the atomic arrangement in ice crystals may eventually be achieved, and a detailed investigation of experimentally induced lattice imperfections also can be carried out.

Acknowledgments

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RADIO-FREQUENCY SPECTROSCOPY OF FROZEN BIOLOGICAL MATERIAL: DIELECTRIC HEATING AND THE STUDY OF BOUND WATER*

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Small bundles of muscle fibers isolated from a muscle that has been frozen and dried will contract when they are rehydrated.[1] This reaction is of interest from two points of view. First, it can be useful in the study of certain aspects of muscle physiology because the water that has been removed can be replaced with solutions having different dielectric constants, and the effect of such replacements upon the contraction can be studied. Second, from the point of view of freeze-drying, the reaction is of interest because it is an example of the effects of this process on a rather complex physiological system and may demonstrate some of the factors influencing the survival of highly complex cells that have been frozen and dried. In addition, some of the methods developed in connection with this study of muscle may be of use in the study of freezing and drying in general.

The Acceleration of Drying by the Use of Dielectric Heating

As an outgrowth of the study of the reaction of water with the dried muscle, an attempt has been made to develop a method for the direct estimation of the binding of water in biological systems. The method is based upon the fact that, in the frozen state, pure ice does not absorb high-frequency radio energy while bound water does so.[2] The usual methods employed in measuring the absorption of radio energy require large samples, and thus are not applicable to material that must be frozen rapidly. The size limitation was overcome by using the resultant heating as an indicator for the amount of energy absorbed. The preliminary data[3] showed that neither pure ice nor dry biological materials absorbed energy at these wave lengths. However, frozen protein solutions or tissues showed a strong heating at temperatures as low as −60° C., which suggested that energy at this wave length might be ideally suited for supplying the latent heat of sublimation during freeze-drying, as suggested by Greaves.[4] This possibility has been investigated by placing the end of the antenna of a radar generator operating at 1000 Mc. around a small histological freeze-drying apparatus at the level of the sample. The heating resulting from the absorption of energy by the sample in the strong field surrounding the antenna was detected with a thermocouple and recorded by a galvanometer. The amount of heating produced in a sample during drying is a function of the rate at which heat is being removed by sublimation, the temperature of the sample, and its composition. The heating produced at a fixed temperature at different times during drying is shown in FIGURE 1. At a pressure of 1 atmosphere, with very little sublimation taking place, there is very marked heating. Early in

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the drying process, when sublimation is occurring at a maximum rate, the heating is greatly reduced. It increases later as the shell of dried material inhibits the rate of sublimation. Finally, when the material is completely dry, there is almost no heating.

Muscle and other tissues dried with the aid of dielectric heating in this manner show no signs of alteration due to the high-frequency radio energy. Cytological examination of these tissues showed them to be the same as other tissues that have been frozen and dried. The muscle behaved in the same way, shortening by the same percentage and developing the same tension as muscle not exposed to the radio energy during drying. Although supplying heat in this way has the marked advantage over heating with infrared radiation of not burning the dried material or heating the container, there are several difficulties that may be encountered in its application. As shown in FIGURES 3 to 7, there are marked differences in heating rates, depending upon the material and its state as well as upon its position in the drying cycle. This means that the temperature must be known so that the power level can be adjusted to prevent overheating or inefficiency due to underheating. Unfortunately, any metallic temperature-sensing element may be heated directly by the radio energy if the proper precautions are not taken, and this may modify the electromagnetic field around the element to produce abnormal heating rates in the immediate vicinity. However, these problems might be avoided by adjusting the power input to produce a certain rate of sublimation, as indicated by the flow of water vapor from the material to the trap, rather than by measuring the temperature of the sample directly. The two difficulties that Greaves suggested might be encountered, namely, ionization discharge and a tendency for the frozen tissue or solutions to explode due to internal heating, have not been observed. However, the sample size has never exceeded 5 cm. in thickness. Such internal heating might possibly occur in pure blocks of ice, but in most biological ma-

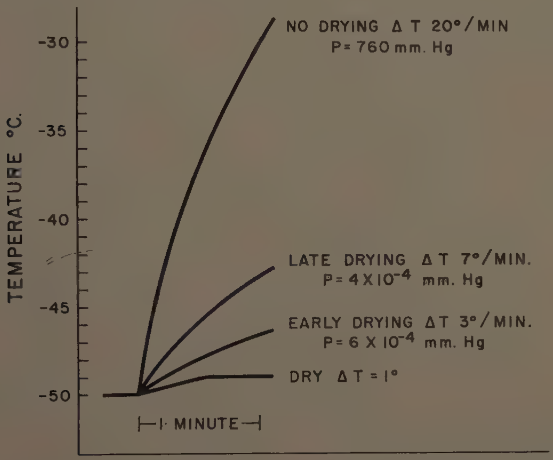

FIGURE 1. Superimposed tracings from a recording galvanometer, showing variation in heating rates of muscle produced by a 1-min. exposure to radio energy at −50° C. at different times in the drying cycle. Power to antenna, 30 w.

terial there is a sufficient number of cracks to allow the escape of vapor. In addition, most of the energy will be absorbed in the outer few centimeters. Perhaps the greatest drawback to accelerating drying through the use of energy at these wave lengths is the factor of cost. The radar tubes available at present are very inefficient, but much more efficient models will be available soon.

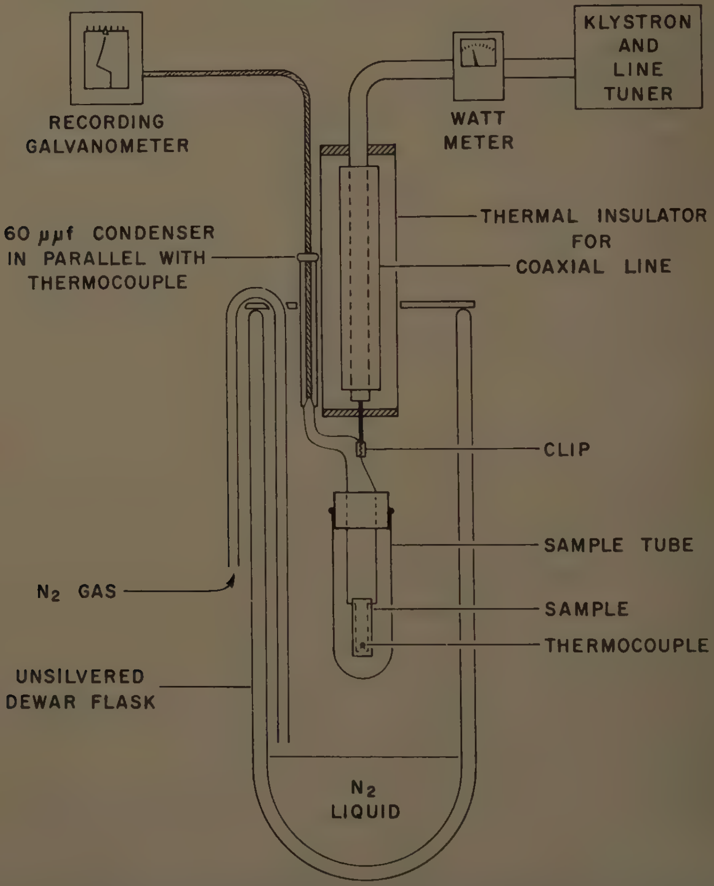

FIGURE 2. Diagram of the apparatus used in these experiments. The power entering the tuned coaxial line was 20 w. Heating of the empty thermocouple is reduced by placing a condenser across the leads. The 1-liter Dewar flask is unsilvered to reduce difficulties in tuning that might result from reflections.

The Estimation of Bound Water by Radio-Frequency Spectroscopy

The further development of the radio-frequency spectroscopy method for estimating bound water has led to some other results that are of interest in the study of freezing and drying. Rather than placing the sample in a field produced by an antenna, as in the preliminary work, it has been found more satisfactory to have the thermocouple carry the energy into the sample. The thermocouple is clipped to a tuned line that has been adjusted so that there is a power maximum at the thermocouple (FIGURE 2). In operation, the power level, tuning, and frequency remain constant. The sample, which need not be larger than 100 mg., is frozen onto the thermocouple and its temperature controlled by adjusting the flow of nitrogen gas entering the liquid nitrogen-cooled Dewar flask. Before each reading the temperature of the sample is stabilized, and the radio-frequency power is then turned on from 5 sec. to 1 min., depending upon the heating rate. These rates, in degrees Centigrade per minute, are plotted as a function of the temperature of the sample at the time the power was turned on. Several points are illustrated in FIGURE 3. Maximum absorption, as indicated by maximum heating, occurs at or above the eutectic temperature of the solution, and the absorption at the lower temperatures is roughly proportional to the ability of the solutes to bind or to prevent complete crystallization of the water. Of particular interest is the fact that the presence of NaCl does not obscure the results below −20° C. The data in FIGURES 4, 5, and 6 give some idea of the complexity of the results that can be obtained with this method and the marked influence of fairly small changes in the physicochemical state of the solution. The method is also sensitive to the thermal history of the sample, as shown in FIGURE 7. Here the only difference is in the rate of freezing; much greater differences are found when other factors in the thermal history are changed. Thus, not only might data of this sort be useful in analysis of the freezing process and factors involved in the survival of frozen cells, but they might also be of use in determining the

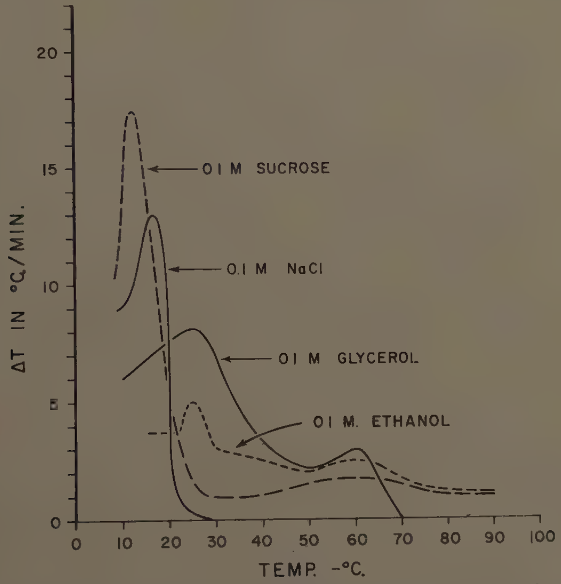

FIGURE 3. Absorption, indicated by heating rates, plotted as a function of temperature. The solutions were held on the thermocouple by hanging over it a strip of glass-fiber filter paper that was then dipped into the solution, withdrawn, and frozen in liquid nitrogen. After 5 min. in liquid nitrogen the temperature of the sample was raised to −100° C., at which point the first readings were made.

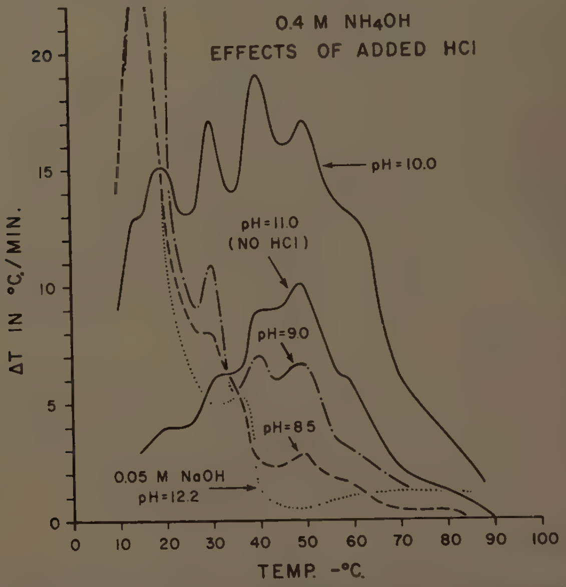

FIGURE 4. Heating rates as a function of sample temperature for 0.4 M ammonium hydroxide solutions at different *p*Hs. Maximum absorption at the lower temperatures occurs in the *p*H region of maximum buffering activity. The *p*H was adjusted with HCl.

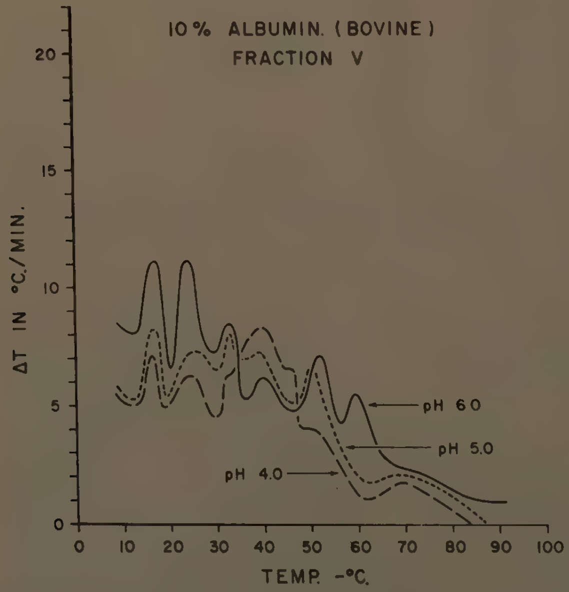

FIGURE 5. Ten per cent albumin in distilled water. The *p*H was adjusted with either HCl or NaOH.

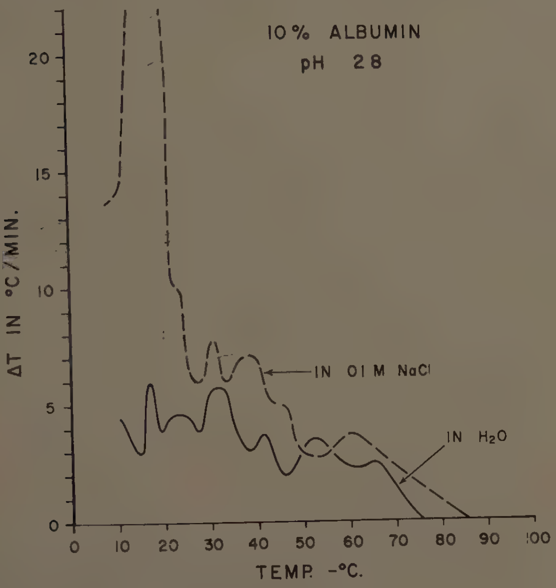

Figure 6. Effects of 0.1 M NaCl on the absorption of a 10 per cent solution of albumin at *p*H 2.8.

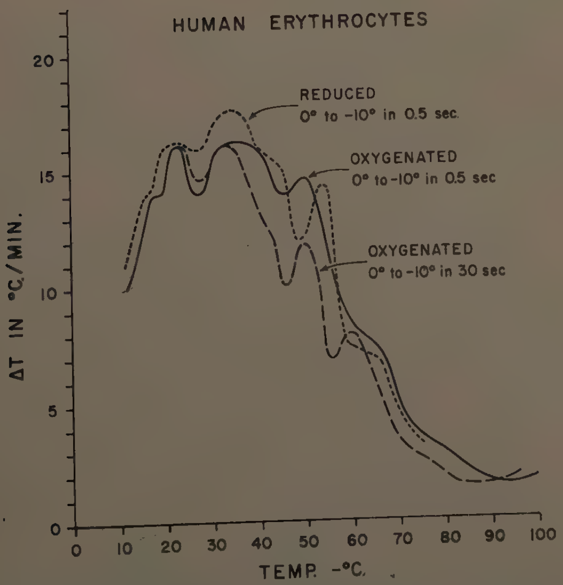

Figure 7. Effect of different rates of freezing upon the radio-frequency absorption of packed human erythrocytes.

effect of the physicochemical state of the material upon the ease with which residual moisture can be removed and the stability of the dry product.

The Detection of Residual Moisture

Early in the work on dry muscle it became apparent that the behavior of muscle on rehydration was greatly affected by the amount of residual moisture it contained. In order to eliminate this variable a very simple but qualitative method was developed for determining the point at which all of the extractable

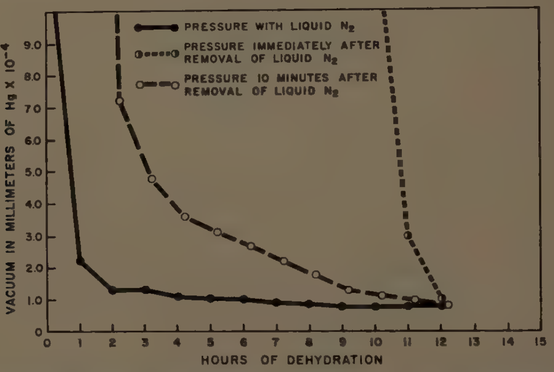

FIGURE 8. Pressure changes during freeze-drying. Only the pressure spike that occurs immediately after the removal of the liquid nitrogen from the cold finger changes with sufficient rapidity to give a clear-cut end point.

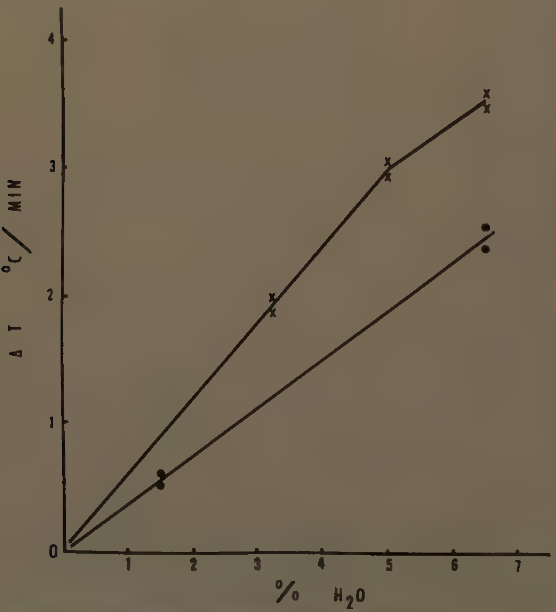

FIGURE 9. The effect of residual water on the dielectric heating of dry crystalline albumin (●) and powdered lactalbumin (X).

water was removed.[5] The drying apparatus was similar in design to that used by Glick,[6] with a cold finger directly over the tissue. Neither the pressure when this finger is full of liquid nitrogen nor the pressure when it is empty shows any sudden change that gives a well-defined end point indicating complete dryness. If at intervals of an hour or more the cold finger is suddenly warmed, there is a sudden increase in the pressure as the accumulated ice sublimates (FIGURE 8). This pressure spike decreases very suddenly and falls to a constant level when the tissue is dry. If the tissue is embedded before it falls, there is bubbling of the paraffin, which indicates that vapor is still escaping from the tissue. However, if embedding is done immediately afterward, there is no bubbling.

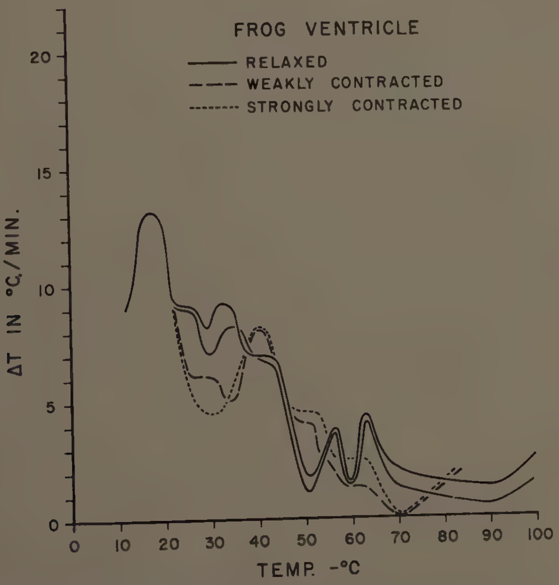

FIGURE 10. Absorption by frog ventricles that were frozen in either the relaxed state (*solid line*) or the contracted state (*broken lines*).

Although this method is satisfactory as an indicator of dryness for many purposes, it does not give any information about how much unextracted water remains in the tissue. In certain cases, particularly in the physicochemistry of proteins, it would be useful to have a method that is sensitive to residual moisture. In this connection, some preliminary data have been gathered on the heating of dry proteins as a function of water content (FIGURE 9). The present equipment is very poorly adapted for this purpose but, with improved design and higher power levels, the method would be quite sensitive. While it would probably be necessary to construct a calibration curve for each type of material, such a method would be advantageous in cases where it was either undesirable to remove all of the water or impossible to do so without damaging the sample.

Significance of the Rehydration Contraction of Muscle to the Survival of Frozen and Dried Cells

There is one aspect of the behavior of frozen dried muscle that is of particular significance from the viewpoint of the survival of complex cells that have been frozen and dried: the fact that the muscle invariably contracts when it is rehydrated. This rehydration contraction, occurring in muscles exposed to concentrations of KCl sufficiently high to prevent the contraction that occurs on freezing, represents quite a large artifact. The most obvious time for the initiation of this reaction is during rehydration, perhaps due to transitory high ionic strength. Recently another possibility has been suggested by the data obtained from the absorption of radio energy by muscle. As may be seen in FIGURE 10, there is a small but definite absorption occurring below −80° C. that actually increases at lower temperatures. The same phenomenon is seen in skeletal muscle, as well.[3] If this absorption is due to the presence of water that is not completely immobilized, it is possible that some degree of artifact formation is occurring during drying even at −80° C., and that it will be necessary to dry at considerably lower temperatures to avoid this effect. Regardless of the origin of the artifact that leads the muscle to contract when rehydrated, it may be necessary to devise some method to control it if complex cells are to survive drying. A great many cells contain structures that have contractile proteins associated with them, and the uncontrolled contraction of these proteins could severely damage parts of the cell. Such damage may be seen to occur in muscle when the rehydration is incomplete or does not occur very rapidly.

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PRESERVATION OF LIVING CELLS BY FREEZE-DRYING

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The preservation of living cells must be the ultimate challenge to most investigators of the problems of freeze-drying. Enormous success has followed the freeze-drying of biological solutions, and it is probably fair to say that the only improvements to be anticipated in this field are of a technical nature, that is, improvements that will increase the speed of drying and decrease its cost.

However, the preservation of living cells by freeze-drying is a much more complex problem. Death may occur either during the freezing procedure or during drying, during storage after drying, or during the period of reconstitution. Survival experiments may not be as easy to interpret as might be imagined; for example, if the addition of some substance to the drying medium improves the survival rate, how are we to tell whether this procedure protected the cell during drying or during reconstitution? The lethal effect of freezing on living cells is well known, but it is not easy to say whether, if death occurred, it did so during the freezing or the thawing. If the latter, it is possible that a freezing procedure that was lethal might still be satisfactory as a preliminary to drying, since the thawing process is thus avoided. Thus the freeze-drying technique becomes of great interest to investigators of the effect of freezing on cells, and the freezing technique becomes of great interest to those studying drying for, when frozen, the free water of the cell becomes immobilized and the cell is dry. Realizing full well the interdependence of one stage on another, it is nevertheless convenient to consider the freeze-drying process under the headings of these stages.

Preliminary Freezing

Cells vary enormously in their sensitivity to freezing and thawing. Briefly, the principal problems of freezing that affect the drying procedure are concerned with the rate of freezing. If freezing is carried out slowly, ice crystals form in the extracellular spaces, and these crystals draw water from the cells, leading to a certain degree of cell plasmolysis. At the same time these crystals may cause considerable deformation of the cells. The plasmolysis of the cells may well be an advantage, as Luyet (1951) discovered when he subjected cells to vitrification. The crushing of the cells by the ice crystals, together with the possibility of their perforation, will cause little or much damage, depending on the type of cell.

More rapid freezing will lead to the formation of small crystals within the cells, and all the evidence indicates that these intracellular crystals are even more lethal than extracellular ones.

Luyet (1951) has shown that, with plasmolysis and ultrarapid freezing, crystal formation may be avoided and delicate cells may be frozen and thawed without injury. Vitrification freezing would therefore seem to be the method of choice for prefreezing before drying. From a practical point of view, the main difficulty is to obtain sufficiently rapid freezing through a reasonable thickness of material. Even if this were achieved, our difficulties would not be ended, for Meryman (1956) has shown that crystals begin to form in pure amorphous ice if the temperature rises to −130° C. and probably at about −100° C. in biological materials. To freeze-dry below −100° C. gives so small a difference in vapor pressure that drying becomes almost impossibly slow and when, in addition, we have the obstruction to vapor flow caused by the cell membrane, the speed of drying is reduced still further.

A promising line of attack is to search for some protective substance. Remarkable results have followed the use of glycerol. From the point of view of drying, it is regrettable that this substance should be nonvolatile, for it concentrates during drying, and we are left with the dried product in anhydrous glycerol. However, this result is probably not very damaging, the injury being caused in the early stages of rehydration. Parkes (1958) has suggested the possible use of methyl alcohol as a substitute for glycerol for drying, but his preliminary results have not been successful.

Drying

Most of the experimental work on the drying of living cells has been done with bacteria and viruses, most of which are reasonably resistant to freezing. I had been drying my stock cultures of bacteria in normal horse serum for several years with what I thought was complete success when it occurred to me that it would save a lot of time preparing mixed plate cultures for teaching purposes if one adjusted the proportions of various organisms and freeze-dried them. When I did this and then plated the specimens, the proportion of the various organisms had been changed completely. Freeze-drying bacterial cultures was not the simple process I had thought it to be. That was 14 years ago, and some of the questions then raised still remain unanswered.

Most of my work has been done with 4 organisms: a coliform bacterium (D201H), chosen because it was sensitive to drying techniques, but not unduly so; the 2 very sensitive organisms *Neisseria gonorrhoeae* and *Vibrio cholerae*, as they were very easy to count; and, finally, BCG, in the hope of producing a very stable vaccine.

Method of drying. Two methods have been in routine use. The first, based on the vacuum spin-freeze method (Greaves, 1944) is used for all routine drying; the second, also described in Greaves (1958), operates with 2 mechanical refrigerators, 1 compressor refrigerating the condenser to −55° C., the other refrigerating the drying chamber to any preset temperature down to −45° C.

Experience has shown that the method of drying used is not of any very great importance. The vacuum spin-freeze method has several limitations, for it cannot be used with drying media containing dextran, which forms a somewhat impermeable skin on the surface when frozen by this method, nor is it very suitable for use with tubes plugged with absorbent cotton, which greatly restricts the flow of vapor, nor can it be used with certain drying media having low-eutectic salt mixtures. The refrigerated machine is more suitable for experimental work, as it allows perfect control and recording of temperatures.

Meryman (1956) has drawn attention to the fact that cells at temperatures just below the freezing point of the solution are especially liable to injury, both from ice crystal growth and from increasing electrolyte concentration, and has suggested that the rate of injury may be far greater at temperatures between 0° and −10° C. on warming from a lower temperature than on initial freezing, which is precisely the state of affairs that exists in most freeze dryers toward the end of the drying cycle. With this in mind Muggleton (1958) asked me to dry some of his BCG vaccine on my refrigerated apparatus at a controlled temperature of −30° C. The 10 per cent survival that he was obtaining on his conventional apparatus rose to nearly 100 per cent on my apparatus. In a series of experiments using a drying medium of 8 per cent dextran, 7.5 per cent sucrose and 2 per cent Na glutamate and drying at temperatures from −10° to −35° C., Muggleton (1958) found that survival increased only slightly down to a temperature of −25° C.; thereafter there was an abrupt increase in survival down to −28° C.

The drying medium. This has proved to be the most important single factor in influencing both the survival rate after drying and the resistance of the dried organism to high temperatures and long storage. The whole subject of the use of adjuvants in drying is a fascinating one but it is very difficult to interpret. Of the many media advocated for drying, those of Naylor and Smith (1946), Hornibrook (1950), and Fry and Greaves (1951) are probably the best known.

In assessing the value of a drying medium, survival tests should be made immediately after drying and after various periods of storage at different temperatures. In my opinion one of these temperatures should be 100° C., for storage at the more usual temperature of 37° C. is so good with modern media that it will be years before the assessment of any change in the medium can be fully known.

Fry and Greaves (1951) introduced a drying medium that they named mist desiccans; it consisted of 75 per cent serum, 25 per cent broth, and 7.5 gm. glucose per 100 ml. The evidence on which this medium was evolved was the following.

Broth had been used for many years with some success for drying organisms, but when Leshchinskaya (1944) suggested drying BCG in 50 per cent aqueous glucose we tested the effect of adding glucose to broth. This procedure improved survival rates enormously, with an optimum at 7.5 per cent. Other sugars, particularly lactose, had similar effects; the curve for sucrose was slightly different, the percentage survival increasing progressively as the concentration increased, although storage survival fell rapidly at concentrations over 10 per cent. The serum was added because it was found to increase the survival rate of *N. gonorrhoeae.* Viable counts of organisms dried in this medium and stored at room temperature have been followed over the last 12 years and are still satisfactory.

We thought at this time that the beneficial effect of glucose was to buffer the final water content of the dried culture, and this has been shown by Ungar *et al.* (1956) to be in the order of 1 per cent residual moisture for 7.5 per cent glucose, thus protecting the organisms from becoming too dry. We supposed that the serum gave some protection to *N. gonorrhoeae* during freezing and also acted as a scaffolding, forming a solid, dried cake.

The work of Scott (1958) probably provides the answer to why broth was such a good drying medium. Scott dried his organisms in a small tube, having suspended them in papain digest broth to which he added various substances. These tubes were then placed in larger tubes containing P_2O_5 or salts at various hydration values. Scott could then store the organisms at various water activities, in vacuum, air, or nitrogen, and at different temperatures. At low water activities and temperatures the high initial count with glucose was well maintained, but there was instability at high temperatures. This disadvantage was largely offset if sucrose was substituted for glucose. However, if ribose was substituted for glucose, storage at all water activities and temperatures was poor. Scott postulates that a fundamental cause of death in dried organisms arises from alteration in cell protein brought about by reaction between the carbonyl compounds present in cells and the amino groups of the cell protein, resulting in the removal of amino side chains. Carbonyl-containing substances such as glucose and, more particularly, ribose, increase the rate of death of microorganisms when stored at high temperatures and water activities. Organisms offering the greatest resistance to death from low concentrations of pentoses were those found to contain free amino acids within their cells. The addition of amino acids to the drying medium vastly improved viability on storage. Thus, we appear to have a reasonable explanation as to why we found the inclusion of broth in mist desiccans so valuable, since it neutralizes the carbonyl groups in the cells and in the glucose. I have confirmed the instability at high temperatures of cultures dried in mist desiccans because the paracolon bacillus is sterilized in 5 min. at 100° C.

I felt at this point that, if stability at high temperatures was to be obtained, it would be necessary to find some substance that would protect the organisms so that they could be dried to a much lower residual water content.

Collier (1955), who dried vaccinia virus in 5 per cent peptone, found that it survived high-temperature storage well, which supports Scott's hypothesis. When I dried the paracolon bacillus in 5 per cent peptone the organisms survived high-temperature storage very much better although the immediate survival was not nearly as good as in mist desiccans; in fact there was good survival after 2 hours at 100° C.

Cho and Obayashi (1956) found that the addition of 1 per cent Na glutamate to the drying medium rendered dried BCG much more stable to high temperatures on storage. Using 5 per cent Na glutamate alone as a drying medium, the paracolon bacillus gave a better immediate survival rate than in 5 per cent peptone, but not so good a high-temperature survival. When, however, the Na glutamate was combined with a colloid such as glucose-free 5 per cent dextran or 5 per cent bovine albumin, a high initial survival was combined with good storage at 100° C.

In contrast to Cho and Obayashi, who added 1 per cent Na glutamate to their BCG-drying medium, I found the optimal concentration to be between 5 and 10 per cent. This figure leads me to think that probably the Na glutamate acts by neutralizing the carbonyl groups within the cell and also as a buffer for the residual moisture content. Muggleton (1958) found that the beneficial effects of Na glutamate were abolished by glucose but not by sucrose, and he recommends a drying medium consisting of 7.5 per cent sucrose and 2 per cent Na glutamate in 8 per cent dextran for drying BCG.

In the present state of our knowledge a drying medium should be compounded as follows:

(1) A support material so that a good, solid, dry cake is formed. Use 5 per cent dextran or 5 per cent bovine albumin.

(2) A buffer to hold the final water content at 1 per cent. Use 5 to 10 per cent sucrose or 5 per cent Na glutamate.

(3) A neutralizer of carbonyl groups. Use 1 per cent Na glutamate if sucrose is used as buffer or 5 per cent Na glutamate if sucrose is omitted.

I find this drying medium very satisfactory for all bacteria and viruses. In my laboratory we are now turning our attention to higher forms of living cells. Our limited experience so far suggests that this medium will not protect cells that are sensitive to freezing.

Secondary drying and sealing. All of the available evidence suggests that a fully dried organism is a dead one. The word dead should be qualified as implying that the organism did not multiply after resolution; this might well be the result of faulty technique. However, if a suitable buffer to control the final moisture content to about 1 per cent is included in the drying medium, a short secondary drying is indicated so that all ampoules have the same moisture content. This secondary drying can be coupled conveniently with vacuum sealing of the ampules.

I have always found vacuum sealing to be more reliable than sealing in nitrogen. This result is probably due to the difficulty of obtaining dry nitrogen that is oil-free. Dry air leads to a rapid fall in viability.

Conclusions

Annear (1956*a*) freeze-dried a 10 per cent peptone, 1 per cent starch, and 7.5 per cent glucose solution to produce freeze-dried plugs in ampules. To each of these he added 1 drop of his culture and then at once connected his ampules to a high-vacuum manifold; there was an immediate foaming of the plugs, the foam drying almost immediately. When he applied this technique to sensitive organisms such as *N. gonorrhoeae* and *V. cholerae*, he obtained survival rates very much higher than any of mine. He also succeeded in drying leptospirae (Annear, 1956*b*) and the protozoon *Strigomonas oncopelti* (Annear, 1956*c*).

Here was a technique that was achieving results far superior to anything achieved by the more usual freeze-drying techniques. Unfortunately, Annear made no temperature measurements, so it is impossible to say whether freezing took place, but the probability is that the thin film of the foam dried very rapidly from the liquid phase.

I therefore suggested to my colleague Mary Burns that she should try Annear's technique for drying the animalcules in pond water. This she did with no success. It was at this time that Meryman (in a personal communication) told me of his success in drying red blood cells and spermatozoa in thin films on nylon mesh. When this technique was tried with pond life, the rotifers and a few paramecia and amoebae survived, but only when no adjuvants were added to the pond water, and when reconstitution was carried out with filtered pond water.

Much further work remains to be done with this method of drying but, taken in conjunction with Meryman's results, this would seem to be a promising line of attack for the drying of more complex forms of living cells.

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DRYING OF LIVING MAMMALIAN CELLS

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One of the irksome aspects of research on the mechanism of freezing injury is the inability to discriminate between the effects of freezing and of thawing when the test is the presence or absence of subsequent viability. When survival is the ultimate measure, freezing and thawing are inseparable. For this reason more than any other, I have been encouraged to undertake an investigation of the freeze-drying procedure, since it provides an alternate pathway by which cells can be returned from the frozen state. The recovery of viable mammalian cells would not have been a study to approach with great optimism had it not been for the successful freezing of blood in the absence of glycerin, since drying from a glycerinated medium leads one into a hopeless situation.

Our early attempts at freeze-drying erythrocytes met with complete failure. Following drying, individual erythrocytes can be seen under the microscope as bright crystalline rings. As isotonic saline is allowed to flow over them, they appear almost instantaneously to swell into spherocytes and burst. Despite the apparent osmotic origin of this rupture, the use of a highly hypertonic reconstituting solution such as 50 per cent sucrose failed to alter the result. It was at this discouraging stage that my associate, Emanuel Kafig, instigated by sheer inspiration, included in his reconstituting medium some clinical dextran, a high-molecular-weight carbohydrate used as a plasma substitute. Much to our amazement, a majority of the cells remained intact and of normal size and appearance following reconstitution with a 40 per cent dextran solution in isotonic saline.

Since that time the freezing, drying, and reconstituting procedures have been extensively varied and investigated; rather than recount the interminable fumblings and missteps of the past two years, I shall summarize the present experimental results and procedures and the theories they have generated.

At first, red cell suspensions were frozen initially by the nitrogen-spray procedure[1] and then transported to a drying chamber for further processing. Following drying it was necessary to crush the dried droplets prior to reconstitution in order that resolvation be rapid. It has since proved simpler and more effective to freeze the cell suspension by sublimation in the same vacuum chamber in which drying will subsequently proceed. The blood suspension is most conviently layered on a sheet of nylon gauze that hangs free in the drying chamber (FIGURES 1 and 2). With the application of reduced pressure from a 27-cu.-foot/min.-capacity mechanical pump, sublimation of water takes place rapidly from the specimen with an associated cooling. With the removal of between 10 and 15 per cent of the specimen water, sufficient cooling is attained to cause freezing. Sublimation and cooling continue following freezing, and equilibrium between radiated heat input from the environment and heat loss from sublimation occurs in our system at −30° C. The rate of sublimation slows appreciably as drying progresses. The temperature rises to −25° C. 3 to 4 min. following freezing and then rises more rapidly to approach room temperature. This simple basic procedure is currently used for all our drying experiments.

Unfortunately we do not as yet have apparatus for measuring residual water in the dried specimens, so that we are unable to give absolute figures for any of the experiments. We can say only that, once the temperature of the specimen has risen above the freezing point, the material is visibly and palpably dry on removal from the vacuum. Mammalian tissues can retain as much as 8 per cent of their water and still appear completely dry, so that we assume that the residual water content has been reduced at least to this level. A cell suspension dried to this degree and no further can be reconstituted with isotonic saline with a recovery of 30 to 50 per cent of the erythrocytes intact. We should note that this degree of dehydration is comparable to that achieved by freezing alone, in which approximately 92 per cent of the water is removed to form ice. Since red cells can be recovered following 92 per cent dehydration

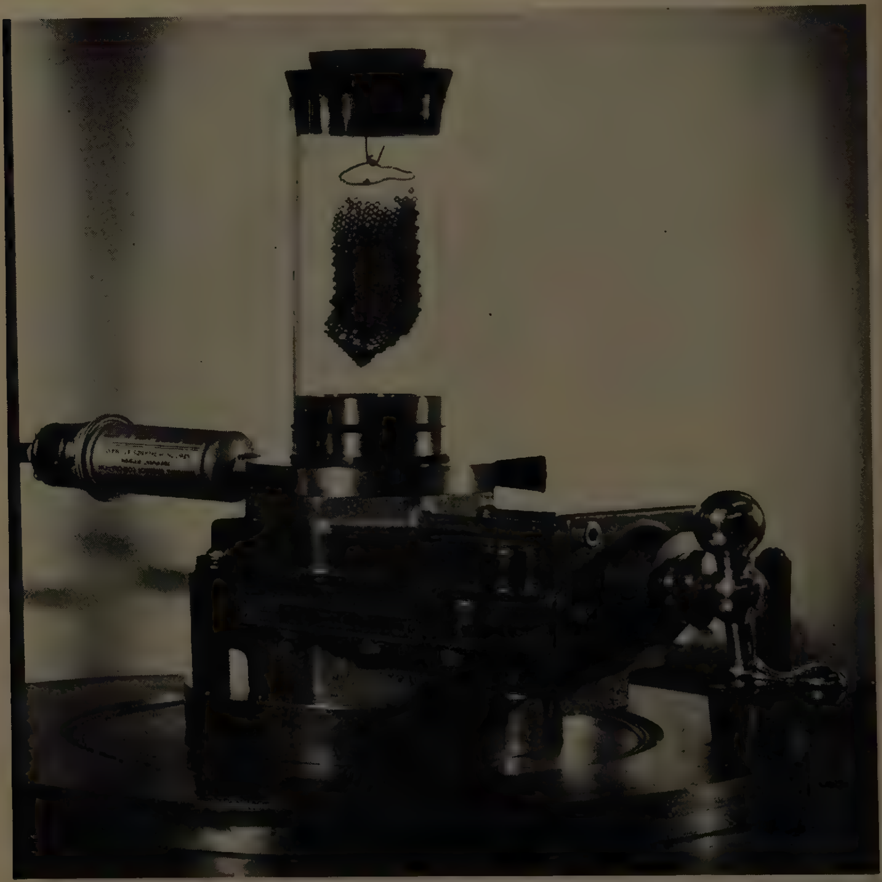

FIGURE 1. Chamber for freeze-drying. Nylon gauze that has been dipped in blood or extended bull semen is hung in a glass chamber over a vacuum valve. The assembly rests over the throat of a large mechanical vacuum pump. The vacuum gauge enters the specimen chamber from the left.

by freezing, we are pleased but not necessarily surprised to find that they can be recovered following a similar degree of dehydration in which the water is completely removed, rather than simply sequestered as ice crystals.

If drying is continued beyond this point until, for example, the specimen temperature has reached ambient, it is no longer possible to recover more than an isolated cell with isotonic saline, and the addition of dextran or the allied polymer, polyvinyl pyrrolidone (PVP), to the reconstituting medium is necessary. The recovery of cells using this reconstituting medium varies, depending upon the degree of dryness and the concentration of the polymer used. When cells are dried only to apparent dryness, up to 100 per cent recovery can be achieved by the use of 40 per cent dextran. An increasing degree of drying or decreasing concentrations of polymer yield reduced recovery. A sample permitted to dry until the temperature has reached ambient would yield from 50 to 60 per cent intact cells with a reconstituting solution containing 20 per cent dextran or PVP. Cells dried in this manner survive in the circulation, as determined in the rat by tagging with chromium-51.

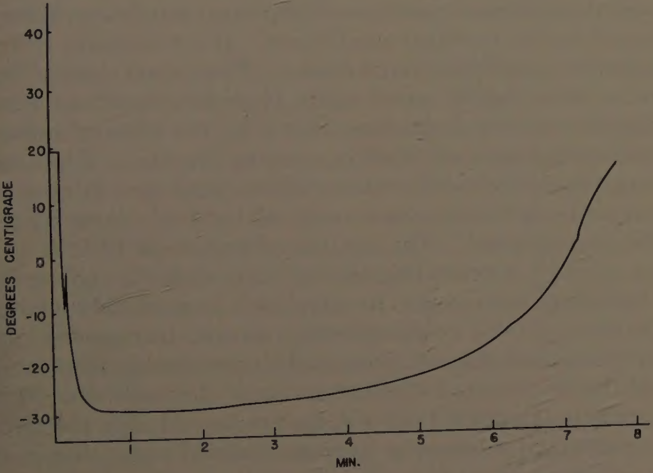

FIGURE 2. Temperature record of bovine semen diluted 20:1 with whole-milk extender; dried with a 27-cu.-ft./min. vacuum pump. This is a typical temperature record obtained during freezing and thawing in the apparatus shown in FIGURE 1. The record is obtained from a copper-Constantan thermocouple of 0.001-in. wire woven into the nylon mesh that supports the specimen. A temperature record for blood would be essentially identical.

If drying is continued still further, the recovery obtainable even with high concentrations of dextran decreases progressively until it is impossible to recover intact cells after an hour or more of drying. However, if such thoroughly dried material is allowed to equilibrate for 24 hours or more in a low water vapor pressure atmosphere such as that over silica gel, recovery is again possible with the use of dextran or PVP. Suspensions of human erythrocytes have been stored in the dry state for periods in excess of 1 year and still permit the recovery of 15 to 20 per cent intact cells upon reconstitution with dextran or PVP. No attempt has been made to measure or control the water vapor pressure of the atmosphere of storage, so that we are limited to the observation that, from the point of view of long-term storage, these are merely encouraging results.

The interpretation of these findings has led us to the following speculation. We assume that the first degree of drying (to 6 or 8 per cent residual water) is comparable to the drying achieved by freezing, represents the removal only of relatively free water, and does not seriously reduce the amount of water involved in the hydration of the organic components. However, further drying begins to remove additional water that is playing a more vital role in the molecular structure of the cell. To explain the protective action of dextran and PVP we are considering the following possibility. If, as has been proposed by others, the transport mechanisms of the cell membrane are contained in holes through the otherwise continuous lipoprotein membrane, these holes are, in fact, plugged by the transport mechanism. It is reasonable to assume that this mechanism is a highly hydrated device. The second stage of drying, with the removal of more tightly bound water, physically disrupts this mechanism by removing its water of hydration, destroying the physical organization of the transport mechanism and, in effect, opening the hole. When cells in such condition are rehydrated with isotonic saline, large quantities of water and externally located solutes can pour into the cell before the integrity of the membrane can be re-established. The inclusion of dextran or PVP in the rehydrating solution provides a protecting blanket that coats the cell and plugs the hole until the membrane recovers its integrity. Immediately following reconstitution the dextran is no longer necessary and can be removed immediately.

Since any mechanism that occupies a hole is presumably retained in position by virtue of the electrophysical configuration of the walls with which it is in close proximity, we can now further speculate that, during the third stage of drying, the more tightly bound or hydrogen-bonded water that is an integral part of the superficial lining of the hole is removed. The configuration of this surface is thus altered to the extent that it can no longer properly support the presence of the plug. Consequently, to rehydrate from this stage it is necessary that the integrity of the hole be first re-established before it can in turn support the transport mechanism that occupies the hole. The replacement of such water may not be possible in a short period of time, and the brief moment of grace provided by the blanketing action of dextran is insufficient. This critical water must be replaced slowly in the absence of sufficient hydration to permit mobility and interaction of proteins and other compounds whose reactive sites have been exposed by the removal of their adsorbed or bound water.

An alternate hypothesis for the reversible last stage of drying is that the last traces of water to be removed come, not from sites with higher binding energies, but from more obscure locations deep within the cell or within macromolecules. To replace such water may require slow preliminary rehydration from the vapor phase.

I have indulged in this perhaps extravagant speculation not so much for its own sake but as an indication of the direction that studies on the freezing and drying of living cells can take. The use of freezing or drying for preserving biological materials is only a portion of its value. In the long run I believe that the greatest benefit from research in this area will be to provide a tool for the study of the role of water in tissues and as a means for suspending transient phenomena for subsequent study.

The demonstration of the resumption of life following freezing or drying is a necessary first step in establishing the research value of these techniques. Once it can be demonstrated that a cell or tissue functions normally following freezing and thawing or freezing and drying we shall have some assurance that its condition while frozen or dried bears some relationship to the living cell. For this reason we are initially obliged, even though only by trial and error, to establish techniques by which viability may be maintained. The erythrocyte is a unique cell, and there is some question as to whether it is viable even in the strictest sense. Therefore, we have turned our attention recently to the spermatozoa, whose habits are such that one can obtain more convincing evidence of its survival.

Bull semen diluted in the conventional extenders used for artificial insemination, egg yolk, and citrate or whole milk, was subjected to the standard procedure already described.[2] It is a tribute to the degree to which intelligence and planning are unnecessary or perhaps even inadvisable for some types of research that we obtained immediate success with the first experiment, which yielded roughly 50 per cent motile spermatozoa following reconstitution with egg yolk or milk extender without any additional components. Subsequent experiments and, in particular, the attempts of others to duplicate this work have demonstrated the remarkable degree of chance involved in this successful experiment. A vacuum pump of approximately one half the capacity of the one we used failed to permit recovery of more than an occasional motile sperm, while one of many times that capacity was equally ineffective. It has developed subsequently that the most desirable combination of circumstances appears to be a relatively slow freezing with as rapid a drying as possible. A lower-capacity pump dries too slowly, while one of higher capacity freezes too rapidly. Diluted bull semen frozen and dried by the standard procedure described has been used* to inseminate a cow. At this writing the expectant mother is entering her third month of pregnancy, and we look forward to a normal and fully hydrated offspring. I must emphasize that this experiment was carried out using bull sperm that was frozen, dried, and reconstituted without an intervening period of storage. Dried sperm stored over silica gel for a period of 24 hours yielded only occasional motile sperm, but storage for an additional 6 days did not decrease this figure significantly. The long-term storage of dried material is quite a different question from the storage of frozen material, where one can predict in advance that a sufficiently low temperature will be indefinitely stabilizing. Many reactions can take place at room temperature, and storage will be possible only if the proper combination of residual hydration, vapor pressure, and perhaps stabilizing additives can be found. To date, we can say only that it is possible, within certain limits, to dry mammalian erythrocytes and spermatozoa and to reconstitute them intact and functioning.

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* Courtesy of the Maryland-West Virginia Artificial Breeders Cooperative, and the University of Maryland, both at College Park, Md.

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